On TRAIL for glioma therapy?

ISBN: 978-90-367-4497-3

Print: Het Grafisch Huis
Cover illustration: Maaike Disco
Layout: Maaike Disco, Aletta de Boer

Rijksuniversiteit Groningen

On TRAIL for glioma therapy?

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 6 oktober 2010
om 13.15 uur

door

Josephus Marie André Kuijlen

geboren op 11 januari 1968
te Luxemburg, Luxemburg

Promotores:	Prof. dr. J.J.A. Mooij Prof. dr. L.F.M.H. de Leij
Copromotores:	Dr. W.F.A. den Dunnen Dr. W. Helfrich
Beoordelingscommissie:	Prof. dr. M.J. Staal Prof. dr. C.M.F. Dirven Prof. dr. H. Hollema

ISBN: 978-90-367-4497-3

‘De trots die je voelt als je vaardigheden toenemen is niet makkelijk te beschrijven. De eerste keer dat je een zestonner het dok invaart en die vervolgens op 25 cm van de kikker legt, het eerste luikdeksel dat je maakt dat geen druppel doorlaat, de eerste reparatie die je succesvol uitvoert- elke keer is het weer een triomf die je leven kleurt geeft. Uiteindelijk wordt het een sport om onafhankelijk te zijn. Je stelt jezelf nieuwe doelen en haalt die.’

(Uit MacBeth J, “Ocean Cruising”. In M. Csikszentmihalyi en I.S Csikszentmihalyi.(eds). Optimal Experience: Psychological Studies of Flow in Consciousness. New York: Cambridge University Press 1988; pp. 214-231)

Contents

Chapter 1

General Introduction

Gliomas comprise the majority of intrinsic cerebral tumors and arise from neuroglial cells. The most common gliomas arise from astrocytes (or precursors) and are called astrocytomas. The World Health Organization has graded them from grade I (benign) to grade IV (malignant) [1]. The grade IV astrocytoma is frequently called glioblastoma (GBM). GBM has a dismal prognosis and after current standard therapeutic regimen of (1) surgery, (2) radiotherapy and (3) chemotherapy with temozolomide, the overall survival reaches a median of only 14.6 months. Although conventional therapies such as maximal safe surgery, radiotherapy, chemotherapy and combinations thereof all contributed to improvement of the overall survival, these treatment modalities seem to have reached a survival plateau. Therefore, there is an urgent need to explore new approaches that can lengthen overall survival of GBM patients.

Nowadays many new anti-cancer approaches are based on extensive knowledge of cellular pathways and new anti-neoplastic agents are developed to interfere within these intracellular pathways on a "targeted" basis. Examples of such targeting strategies are: -1) blocking the EGF receptor (EGFR) with anti-EGFR monoclonal antibodies (Cetuximab) thereby inhibiting EGFR signaling; - 2) disrupting the receptor catalytic activity and inhibiting EGFR autophosphorylation and downstream signaling by targeting the intracellular catalytic domain of EGFR tyrosine by tyrosine kinase inhibitors (erlotinib and gefitinib); - 3) the use of antisense molecules inhibiting translation of the EGF; - 4) the use of small molecule inhibitors against anti-apoptosis proteins, such as Bcl-2 and Bcl-X_L; - 5) inhibiting transcription of several anti-apoptotic proteins (IAP's) by histone deacetylase inhibitors in order to bypass resistance to apoptotic signaling; - 6) inducing apoptosis by Tumor Necrosis Factor (TNF) or its related cytokines.

In this thesis we focus on a TNF related apoptosis inducing ligand (TRAIL) which can be targeted to its cognate death receptors, thereby activating the so-called extrinsic apoptotic pathway. In general, apoptosis is a biological regulative process for an organism to dispose needless or dangerous cells, without causing damage to the surrounding environment. This distinctly differs from necrosis, which is an uncontrolled process that leads to inflammation and in the brain to cerebral edema.

The death inducing ligand, TRAIL can be considered a new non-conventional anticancer drug, which has the potential to induce apoptosis in glioma cells without being neurotoxic. TRAIL (also called Apo2L) is a member of the tumor necrosis factor (TNF) cytokine family which includes FasL (CD95 L) and TNF-α. TRAIL induces apoptosis after crosslinking the death inducing receptors TRAIL-R1 or TRAIL-R2 and thereby activating the extrinsic pathway. In contrast, chemotherapy and radiotherapy activate the intrinsic pathway through DNA damaging.

TRAIL is present as an endogenous cytokine in the human body but its physiological role is not yet fully understood. Functionally, TRAIL has shown to selectively kill tumor cells, while normal cells are left unharmed, this in contrast to TNF-α which is

highly hepatotoxic. In vitro, and animal in vivo studies have shown potent apoptosis inducing activity towards various tumor cell lines including GBM cell lines and as such TRAIL has gained substantial research interest during the last decade. In this thesis several aspects of TRAIL receptor-mediated apoptosis induction in GBM are described and possible opportunities to exploit TRAIL-derivatives against primary brain tumors are discussed.

Aims and outline of the thesis

In order to determine if TRAIL is applicable as an anti-neoplastic agent in patients with a GBM knowledge on the presence of TRAIL receptors in malignant astrocytic tumors or normal tissues is important. Also understanding how and to what extent TRAIL induces apoptosis in GBM is essential. Therefore, the mechanism of TRAIL induced apoptosis must be understood. Understanding of the apoptosis pathways in glioma cells is important to predict sensitivity of these tumors for TRAIL. Resistance of GBM to TRAIL can impede the therapeutic efficacy of TRAIL. This resistance may be the result of defects in the apoptosis signaling pathways. Detailed knowledge on defects in the apoptosis pathways in GBM cells may be useful to predict sensitivity for TRAIL-based approaches.

Chapter 2 represents a review on TRAIL biology and elaborates on the presence of TRAIL receptors in normal tissues and gliomas. Beneficial effects of combinational treatment of TRAIL with conventional therapies are discussed. Resistance pathways of tumor cells for TRAIL and possible solutions to overcome TRAIL resistance are discussed. Moreover, novel approaches to selectively target soluble TRAIL to pre-selected tumor-associated antigens of cancer cells are reviewed.

Data on the presence of death inducing TRAIL receptors on primary GBM cells is sparse[2,3]. Research done on glioma cell lines, which differ for primary tumor tissue, show the expression of both death inducing receptors TRAIL-R1 and TRAIL-R2[4]. However if TRAIL is to be used as anticancer drug for GBM it is important that TRAIL receptors are present on primary GBM cells. Therefore the question arises: are TRAIL receptors present on primary GBM tissue and does the amount of expression correlates with survival? **In Chapter 3** the semi-quantitative and quantitative expression of TRAIL receptors on primary GBM tissue and its association with patient survival are described.

In Chapter 4 the novel development of a TRAIL fusion protein (scFv54:sTRAIL) with specificity for the epidermal glycoprotein 2 antigen is described. “Targeting” drugs to the site of the tumor is a principle by which a drug is specifically directed to tumor associated antigens, present on the cell membrane of the tumor cells. The rationale

is to deliver an optimal dose of drugs at the site of the tumor. Furthermore, *in vitro* studies have shown that TRAIL-R1 has distinct other properties than TRAIL-R2. It has been shown that TRAIL-R2 can only be activated by multiple TRAIL molecules which are interconnected with each other (crosslinked) by a crosslinking enhancing tag. In contrast TRAIL-R1 can be activated by both non-crosslinked and crosslinked TRAIL. However it has been shown that tagged-TRAIL can alter the structure of the receptor resulting in hepatotoxicity. Therefore TRAIL alternatives have been developed with the capacity to be specifically targeted to the tumor cell and also having the capacity to crosslinking TRAIL receptors thereby activating both the TRAIL-R1 and TRAIL-R2 receptor. Questions addressed were; is it possible to engineer a TRAIL fusion protein with targeting activity toward a tumor associated antigen and does this specific targeting lead to an enhanced death inducing capacity? Also the possible advantages of tumor-selective targeting of TRAIL are discussed in chapter 4.

Systemic application of TRAIL has the disadvantage that TRAIL must cross the blood brain barrier in order to eliminate tumor cells. It is questionable whether systemic administration of TRAIL will lead to a relevant concentration in the tumor and the peritumoral region. A possible method to bypass this problem is delivering TRAIL intracerebrally, preferentially within the tumor or the peritumoral area. A technique for delivering TRAIL in the vicinity of the tumor is through intracerebral implantation of encapsulated TRAIL producing cells. Studies concerning the application of alginate encapsulated producer cells to cure neurodegenerative diseases and brain tumors have been published with various success rates[5-10]. Insufficient biocompatibility of the capsules and subsequent death of the encapsulated producer cells has hampered the success and clinical application of this technology. Recent advances such as application of pure alginates with a defined composition has brought new insight in the factors determining the biocompatibility of the capsules. In Chapter 5 a newly developed fusion protein “scFv425-sTRAIL” was used. The manufacturing of scFv425-sTRAIL producing cells and their microencapsulation in pure alginates was evaluated. Also the biological properties of the TRAIL producing cells after encapsulation were assessed. A mouse brain model was used to evaluate the biocompatibility of the alginate capsules after intracerebral implantation. Furthermore the efficacy of alginate encapsulated scFv425-sTRAIL producing cells and the potential of this method for targeted delivery of a TRAIL fusion protein to brain tumor cells is discussed in **chapter 5**.

Another method to deliver drugs to the site of the tumor is the convection enhanced delivery (CED) technique. CED of drugs to the site of the tumor is an extensively studied method. It has been proven effective in several in vitro and animal in vivo studies. Several Phase III studies addressing the issue to target toxins with CED to GBM were initiated (multicenter trial Phase III, PRECISE study; CED of IL13-Pseudomonas exotoxine intracerebrally and phase III study TransMID; Tf-CMR107; Transferrin-

Diphteria tox). Based on the results in the literature it seems logical to explore the possibilities of convection enhanced delivery of a TRAIL fusion protein targeted against the EGF receptor (scFv425:sTRAIL) in a murine brain tumor. Therefore we needed to address the following issues; can we identify a cell line which is extremely sensitive to the scFv425:sTRAIL fusion protein? Can this cell line be implanted in a mouse brain resulting in a high probability of acceptance? If we use a CED technique for delivering the TRAIL fusion protein does it show any efficacy in a certain mouse brain tumor model? In **Chapter 6** these questions are considered and partially answered.

Currently, the standard treatment for patients with a GBM is surgery followed by radiotherapy and chemotherapy. Although this therapy scheme has lengthened survival, still there are glioma cells which show resistance or acquire resistance to either therapy. Also resistance of glioma cells to TRAIL is a know feature. It has been suggested, in non-GBM cell lines, that radiation can enhance the apoptosis-inducing efficacy of TRAIL[11-16]. Whether or not this is a general effect seen in all TRAIL receptor-positive cell lines and whether or not this also enhances the ultimate loss of clonogenicity of tumor cells remains to be elucidated. It would be interesting to evaluate if TRAIL in combination with radiotherapy would lead to enhanced apoptosis in cancer cells thereby bypassing resistance and leading to prolonged survival. In **Chapter 7** we evaluated the effect of combined α-radiation-TRAIL therapy in a glioblastoma cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints.

A general discussion is given in **Chapter 8**, followed by some thoughts on future perspectives. **Chapter 9** summarizes the thesis.

References

1. Pathology & Genetics of Tumours of the Nervous System. Lyon: International Agency for Research on Cancer, 2000,
2. Frank S, Kohler U, Schackert G, et al: Expression of TRAIL and its receptors in human brain tumors. Biochem Biophys Res Commun 257:454-459, 1999
3. Rieger J, Ohgaki H, Kleihues P, et al: Human astrocytic brain tumors express AP02L/TRAIL. Acta Neuropathol (Berl) 97:1-4, 1999
4. Knight MJ, Riffkin CD, Muscat AM, et al: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. Oncogene 20:5789-5798, 2001
5. Emerich DF, Winn SR, Hantraye PM, et al: Protective effect of encapsulated cells producing neurotrophic factor CNTF in a monkey model of Huntington's disease. Nature 386:395-399, 1997
6. Joki T, Machluf M, Atala A, et al: Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol 19:35-39, 2001
7. Mitsumoto H, Ikeda K, Klinkosz B, et al: Arrest of motor neuron disease in wobbler mice cotreated with CNTF and BDNF. Science 265:1107-1110, 1994
8. Read TA, Farhadi M, Bjerkvig R, et al: Intravital microscopy reveals novel antivascular and antitumor effects of endostatin delivered locally by alginate-encapsulated cells. Cancer Res 61:6830-6837, 2001
9. Thorsen F, Read TA, Lund-Johansen M, et al: Alginate-encapsulated producer cells: a potential new approach for the treatment of malignant brain tumors. Cell Transplant 9: 773-783, 2000
10. Winn SR, Wahlberg L, Tresco PA, et al: An encapsulated dopamine-releasing polymer alleviates experimental parkinsonism in rats. Exp Neurol 105:244-250, 1989
11. Zhou Q, Fukushima P, DeGraff W, et al: Radiation and the Apo2L/TRAIL apoptotic pathway preferentially inhibit the colonization of premalignant human breast cells overexpressing cyclin D1. Cancer Res 60:2611-2615, 2000
12. Di Pietro R, Secchiero P, Rana R, et al: Ionizing radiation sensitizes erythroleukemic cells but not normal erythroblasts to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)--mediated cytotoxicity by selective up-regulation of TRAIL-R1. Blood 97:2596-2603, 2001
13. Kim MR, Lee JY, Park MT, et al: Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. FEBS Lett 505:179-184, 2001
14. Ramp U, Caliskan E, Mahotka C, et al: Apoptosis induction in renal cell carcinoma by TRAIL and gamma-radiation is impaired by deficient caspase-9 cleavage. Br J Cancer 88:1800-1807, 2003
15. Chinnaiyan AM, Prasad U, Shankar S, et al: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 97:1754-1759, 2000
16. Gong B, Almasan A: Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. Cancer Res 60:5754-5760, 2000

Chapter 2

On TRAIL for malignant glioma therapy?

Jos MA Kuijlen[1], Edwin Bremer[2], Jan Jakob A Mooij[1],
Wilfred FA den Dunnen[3] and Wijnand Helfrich[4]

[1]Department of Neurosurgery, University Medical Center Groningen, The Netherlands
[2]Department of Molecular Internal Medicine, Medical Clinic and Polyclinic II, University of Wuerzburg, Germany
[3]Department of Pathology and Medical Biology, University Medical Center Groningen, The Netherlands
[4]Department of Surgery, Surgical Research Laboratories, University Medical Center Groningen, The Netherlands

Neuropathology and Applied Neurobiology 2010; 36:168–182

Abstract

Glioblastoma Multiforma (GBM) is a devastating cancer with a median survival of around 15 months. Significant advances in treatment have not been reached yet, even with a host of new therapeutics under investigation. Therefore, the quest for a cure for GBM remains as intense as ever. Of particular interest for GBM therapy is the selective induction of apoptosis using the pro-apoptotic Tumor Necrosis Apoptosis Inducing Ligand (TRAIL). TRAIL signals apoptosis via its two agonistic receptors TRAIL-R1 and TRAIL-R2. TRAIL is normally present as homotrimeric transmembrane protein, but can also be processed into a soluble trimeric form (sTRAIL). Recombinant sTRAIL has strong tumoricidal activity towards GBM cells, with no or minimal toxicity towards normal human cells. Unfortunately, GBM is a very heterogeneous tumour, with multiple genetically aberrant clones within one tumour. Consequently, any single agent therapy is likely to be not effective enough. However, the anti-GBM activity of TRAIL can be synergistically enhanced by a variety of conventional and novel targeted therapies, making TRAIL an ideal candidate for combinatorial strategies. Here we will, after briefly detailing the biology of TRAIL/TRAIL-receptor signalling, focus on the promises and pitfalls of recombinant TRAIL as a therapeutic agent alone and in combinatorial therapeutic approaches for GBM.

Introduction

Glioblastoma multiforme (GBM) is the most frequent and aggressive type of tumour to develop from neuro-epithelial tissue. GBMs are very heterogeneous with multiple clones that contain varied genetic imbalances within one tumour, making it a very hard cancer to treat successfully. Even with improved surgical techniques and post-operative radiotherapy, the mean overall survival time of patients with GBM after neurosurgical debulking and radiotherapy is still limited to approximately 12 months. Importantly, most chemotherapeutics have no real beneficial effect on patient survival [1,2,3,4]. The only positive exception is the alkylating agent Temozolomide (TMZ), which in combination with radiotherapy prolongs survival by 2 to 3 months and doubles the number of long-term survivors [5]. However, it is painfully obvious that the treatment options of the clinician are at the moment ineffective for GBM. Therefore, development of new and more potent therapies is urgently needed.

In recent years, a variety of cancer-specific molecular aberrations have been identified and subsequently exploited as potential targets for the treatment of patients with GBM therapy. A particularly promising novel therapeutic approach for GBM is the reactivation of apoptosis using members of the Tumour Necrosis Factor (TNF) family, of which the TNF-related Apoptosis-Inducing Ligand (TRAIL) holds the greatest appeal. TRAIL is an effector molecule involved in immune surveillance by various T cell subpopulations and NK-cells. TRAIL is important for the elimination of virally-infected and cancer cells [6,7,8]. Apoptotic activity of TRAIL towards normal cells appears very limited, if present at all. By now a recombinant version of TRAIL has advanced into clinical trials for Chronic Lymphocytic Leukemia, with promising preliminary data on tolerability and beneficial therapeutic activity. Both the organized way of getting rid of malignant cells by apoptosis in combination with the lack of neuro or systemic toxicity makes TRAIL an interesting molecule to treat GBM. In this review, we first detail TRAIL-TRAIL receptor biology after which the potential of TRAIL-based therapeutics for the treatment of GBM will be discussed.

TRAIL/TRAIL-receptor biology

TRAIL is normally expressed on both normal and tumour cells as a non-covalent homotrimeric type-II transmembrane protein (memTRAIL). In addition, a soluble form of TRAIL (sTRAIL) can be generated due to alternative mRNA splicing [9] or proteolytic cleavage of the extracellular domain of memTRAIL [10,11] and thereby still retaining tumour-selective pro-apoptotic activity [12,13].

TRAIL has an intricate receptor system comprising 4 distinct membrane receptors, designated TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4. Of these receptors, only TRAIL-R1 and TRAIL-2 transmit the apoptotic signal. These two receptors belong to a subgroup of the TNF receptor family, the so-called Death Receptors, and contain the hallmark intracellular death domain (DD). This DD is critical for apoptotic signalling by Death Receptors.

TRAIL activates the extrinsic pathway of apoptosis by binding to TRAIL-R1 and/or TRAIL-R2 (Figure 1), whereupon the adaptor protein FADD and initiator caspase-8 are recruited to the DD of these receptors. Assembly of this so-called Death Inducing Signalling Complex (DISC) leads to the sequential activation of initiator and effector caspases, and ultimately results in apoptotic cell death.

In certain cells, the execution of apoptosis by TRAIL further relies on an amplification loop via the intrinsic mitochondrial pathway of apoptosis. The mitochondrial pathway of apoptosis is a stress-activated pathway, e.g. upon radiation, and hinges on the depolarization of the mitochondria, leading to release of a variety of pro-apoptotic factors into the cytosol (Figure 2). Ultimately, this also triggers effector caspase activation and apoptotic cell death. This mitochondrial release of pro-apoptotic factors is tightly controlled by the Bcl-2 family of pro- and anti-apoptotic proteins [14]. In the case of TRAIL-receptor signalling the Bcl-2 Homology (BH3) only protein Bid is cleaved into a truncated form (tBid) by active caspase-8. Truncated Bid subsequently activates the mitochondrial pathway.

TRAIL-R3 is a GPI-linked receptor that lacks an intracellular domain, whereas TRAIL-R4 only has a truncated and non-functional DD. The latter two receptors are thought to function as decoy receptors that modulate TRAIL-sensitivity, however the mechanism underlying this decoy function is not yet elucidated. Evidence suggests that TRAILR3 binds and sequesters TRAIL in lipid membrane microdomains. TRAIL-R4 appears to form hetero-trimers with TRAIL-R2, whereby TRAIL-R2 mediated apoptotic signalling is disrupted. TRAIL-R4 might activate Nuclear Factor kappa B (NFkB), although conflicting evidence concerning activation of NFkB exists [15,16]. Of note, TRAIL also interacts with the soluble protein Osteoprotegerin (OPG), although the exact consequence of this interaction remains to be clarified.

TRAIL-(receptor) expression in human tissues

Although data on the expression of TRAIL in normal human tissues is limited, diffuse expression of TRAIL has been detected on liver cells, bile ducts, tubuli contorti of the kidney, cardiomyocytes, lung epithelia, Leydig cells, normal odontogenic epithelium, megakaryocytic cells and erythroid cells[17,18,19,20]. In contrast, no or weak expression of TRAIL was in colon, glomeruli, Henle's loop, germ and Sertoli cells of the testis, endothelia in several organs, smooth muscle cells in lung, spleen and in follicular cells in the thyroid gland[21,22]. With regard to the brain, expression of TRAIL has been detected on mRNA levels in normal brain tissue (not specified if this was neuronal or glial tissue) as well as protein level (glial cells of the cerebellum) [23,22]. One study was unable to confirm these findings [24].

All of the TRAIL receptors are expressed on many normal tissues. For instance, TRAIL-R1 is expressed on heart myocytes, colon, lung epithelium, Leydig cells, ovaries and in the brain on astrocytes[25,17,24]. TRAIL-R2 is detected on heart myocytes, colon, lung epithelium, Leydig cells, ovaries, oligodendrocytes and on neurons [25,17,24]. Vascular brain endothelium appears to be negative for TRAIL-R1 and weakly positive for TRAIL-R2 [17]. With regard to the decoy receptors, TRAIL-R4 and TRAIL-R3 have been detected on oligodendrocytes and neurons[24].

TRAIL-receptor expression in glioblastoma

TRAIL-R1 and TRAIL-R2 are ubiquitously expressed on a variety of tumour types [26,25,27,17,21,28]. Importantly, TRAIL-R1 and TRAIL-R2 are also expressed in the tumour tissue from astrocytoma grade II and glioblastoma patients[23]. In a study on 62 primary GBM tumour specimens, TRAIL-R1 and TRAIL-R2 were expressed in 75% and 95% of the tumours respectively. Of note, a statistically significant positive association was identified between agonistic TRAIL receptor expression and survival [29]. Interestingly and perhaps counter intuitively, highly malignant tumours actually express a higher amount of TRAIL receptors in comparison to less malignant tumours or normal tissue. In general TRAIL-R2 is more frequently expressed on tumour cells than TRAIL-R1. Several studies in GBM cell lines were unable to correlate TRAIL-sensitivity to the expression levels of the agonistic TRAIL receptors TRAIL-R1 or TRAIL-R2 nor to the expression levels of the decoy receptors TRAIL-R3 and TRAIL-R4 [30,31].

Recombinant TRAIL and TRAIL-R specific antibodies for tumour therapy

TRAIL and agonistic antibodies directed at the TRAIL death receptors TRAIL-R1 and/or TRAIL-R1 hold a prominent place as potential anti-cancer drugs[32,33,34]. Indeed, many

tumour types are sensitive to apoptotic elimination by TRAIL, whereas normal human cell types are resistant. A variety of sTRAIL preparations has shown promising tumoricidal activity *in vitro* and *in vivo*. Importantly, loco-regional application of TRAIL in an intracranial GBM xenograft model of the cell line U87MG revealed strong tumoricidal activity towards pre-established xenografts, with long-term survival of >100 days in treated mice compared to ~36 days survival in non-treated mice.

These preclinical studies have illustrated the promise of TRAIL as a therapeutic reagent *in vivo* with no or minimal toxicity. Indeed, a recombinant trimeric form of TRAIL is being explored in an ongoing multi-centre clinical trail for B-Chronic Lymphocytic Leukemia patients. Preliminary reports indicate that the main effect of single agent TRAIL treatment in CLL patients is the induction of stable disease and a number of partial responses at higher doses of 8 mg/kg[35,32]. Importantly, no significant side-effects have been reported so far, thus corroborating the apparent safety of sTRAIL treatment in humans.

In addition, a number of agonistic antibodies (HGS-ETR1, HGS-ETR2, HGS-TR2J, LBY135, CS-1008, AMG 655) that selectively target TRAIL-R1 or TRAIL-R2 have been developed. All of these antibodies have potent tumoricidal activity *in vitro* and *in vivo* and appear to have a low toxicity profile in early phase clinical studies [36,33,37,38,39]. An obvious difference between these TRAIL-receptor-selective mAbs and TRAIL is the fact that TRAIL interacts with both of its agonistic receptors. This might provide TRAIL either with a wider therapeutic spectrum or a narrow and more unpredictable therapeutic window, especially in light of its additional interaction with decoy TRAIL receptors.

It is interesting to note that several groups have pursued the design of sTRAIL variants that show selectivity for TRAIL-R1 or TRAIL-R2[40,41,42,43]. Although the precise fine-specificity of some of these variants remains a matter of debate, the use of TRAIL-receptor selective variants for the treatment of a specific tumour type may prove valuable. For instance, CLL appears to be preferentially sensitive to TRAIL-R1 apoptotic signalling, whereas certain solid tumours appear to preferentially signal via TRAIL-R2. Rational integration of TRAIL-receptor selective sTRAIL variants may in those cases help to optimize efficacy.

Importantly, as will be described in more detail below, normal cells can be sensitized to sTRAIL by certain other anti-cancer drugs. These side effects are likely due to a sensitizing effect by the co-administered drug on normal cells for the ubiquitous priming of TRAILR1 by sTRAIL trimers, since sTRAIL trimers are fully capable of TRAILR1 activation. In contrast, TRAILR2 is not/minimally activated by homotrimeric sTRAIL. Therefore, it seems a reasonable assumption that TRAILR1 signalling is the main culprit behind potential side effects of sTRAIL trimers. Thus, the rational design and use of TRAIL-R2 selective sTRAIL variants may help to optimize therapeutic efficacy, while minimizing the occurrence of toxic side-effects.

TRAIL-resistance in GBM

The available preliminary data indicate that activation of apoptotic TRAIL-receptor signalling using sTRAIL or agonistic TRAILR antibodies may indeed prove beneficial to cancer patients and certainly warrant further evaluation of this reagent in clinical trials. However, intrinsic and/or acquired resistance to TRAIL-receptor signalling is likely to pose a significant hurdle to clinical efficacy. Indeed, almost half of tumour cell lines analyzed have intrinsic resistance to TRAIL-receptor signalling, which also holds true for GBM cell lines. Resistance of GBM to TRAIL may be due to a variety of reasons, including high decoy receptor expression, low expression levels of critical mediators of TRAIL-signalling, such as caspase-8 and FADD [30], or high expression of inhibitors of apoptosis such as cellular-Flice Inhibitory Protein (cFLIP)[44-46]. Interestingly, a recent report indicates that non-genetic naturally occurring differences in the levels or states of anti- or pro-apoptotic proteins are the primary causes of cell-to-cell variability in timing and likelihood of apoptotic cell death in cell lines [47]. Of note, TRAIL-resistance seems to be even more pronounced when assessing TRAIL activity towards primary patient material. Indeed, TRAIL sensitivity in GBM cell lines does not correlate well with activity towards primary GBM cells. In fact, TRAIL resistance in primary GBM cells appears rather widespread, thus questioning the ultimate clinical benefit of TRAIL as single agent therapy.

Overcoming TRAIL-resistance by combination therapy with radiation/chemotherapy

Intrinsic or acquired resistance to TRAIL can often be overcome by combination of TRAIL-based agents with chemotherapeutics, radiation or other novel therapeutic drugs. Preliminary clinical data also highlights the rationale of this approach, with 2 complete and 2 partial responses upon co-treatment of a small group of non Hodgkin lymphoma (NHL) patients with TRAIL and the anti-CD20 antibody rituximab [48]. These clinical observations are corroborated by recent *in vitro* data indicating that combined treatment of cells with rituximab and TRAIL or an agonistic TRAILR1 antibody synergistically induced apoptosis[49,50]. Thus, the presence of *in vitro* synergy may be a useful indicator for potential clinical benefit in combinatorial strategies.

Both radiotherapy and chemotherapy have been studied in combination with TRAIL in preclinical studies in a variety of tumour types [51-62]. With regard to GBM, positive results on tumour regression were obtained after combination therapy. This synergy may be due to various points of crosstalk between TRAIL and chemo/radiation (for overview see figure 3, pg 184) including upregulation of agonistic TRAIL receptors by irradiation [56-58] and chemotherapy [59]. Of note, upregulation of TRAIL-R2 by chemotherapeutics in TRAIL resistant GBM cell lines appears to be p53 dependent, with upregulation of TRAILR2 only occurring in p53wt but not p53mut cells[60]. In contrast, others have found no effect on the level of receptor expression after irradiation or chemotherapy [51,61]. As p53 mutations

are mainly associated with secondary GBM's while EGFR amplification and mutation is strongly associated with "de novo" GBM it should be considered that bypassing resistance, for both GBM subgroup tumors, requires different targeting strategies.

Another possible point of synergy is down-regulation of the anti-apoptotic proteins cFLIP and phosphoprotein enriched in Diabetes/Astrocytes (PED/PEA-15) that both competitively inhibit caspase-8 activation in the DISC[63].

Systemic in vivo administration of TRAIL with cisplatin synergistically suppressed both tumour formation and growth of established subcutaneous human glioblastoma xenografts in nude mice and also significantly extended the survival of mice bearing intracerebral xenografts compared to single agent treated mice [59]. In another study, the efficacy of intra-cerebral infused TRAIL was significantly enhanced by co-treatment with TMZ in a U87MG intracranial xenograft model [62]. As TMZ is nowadays part of the standardized treatment schedule of patients with GBM it will be in the future used in combination with several other drugs for example TRAIL.

Overcoming TRAIL-resistance by combination therapy with new 'smart' drugs

Preclinical studies have also evaluated the combination of sTRAIL with a variety of novel therapeutic approaches for potential synergistic pro-apoptotic activity (for overview see figure 3, pg 184). The results of all these studies clearly demonstrate the added benefit of combination therapy on TRAIL-mediated cytotoxicity. Of particular interest for GBM is the combination treatment of cells with TRAIL and proteasome inhibitor Bortezomib. Bortezomib inhibits the Ubiquitin-proteasome pathway (UPP), which controls the timely removal and degradation of the majority of cellular proteins[64]. An important feature of bortezomib is the differential response of normal and cancer cells to treatment[65]. Both normal and cancer cells are growth-arrested in the G2/M phase of the cell cycle. However, whereas cancer cells die by apoptosis, normal cells resume division after treatment. Bortezomib has been shown to potently augment the apoptotic activity of other therapeutics, including TRAIL[66]. Notably, primary TRAIL-resistant GBM cells were highly sensitive to combination treatment with bortezomib and TRAIL[63].

Another interesting candidate is the antibiotic rapamycin, which inhibits the pro-survival Akt-mTOR pathway by inhibiting mTOR. Akt pro-survival signalling is often up-regulated in glioblastoma and therapeutic inhibition appears warranted. Importantly, rapamycin sensitizes cells to TRAIL-mediated apoptotic signalling. The Akt-mTOR pathway is causally linked to PTEN status of glioblastoma cells, which may be used to enable the identification of a subset of patients that would benefit from rapamycin-TRAIL combination therapy[67]. Also X-linked inhibitory apopototic proteins (XIAP) antagonists are used in combination with TRAIL. Clinical studies with antisense oligonucleotide targeting

X-linked IAP's (XIAP) are ongoing[68].

As described above, the intrinsic mitochondrial pathway of apoptosis is regulated by the balance between pro- and anti-apoptotic members of the Bcl-2 family[14]. In GBM, anti-apoptotic proteins such as Bcl-2 are frequently overexpressed, leading to cell survival. Selective inhibition of these anti-apoptotic proteins has been successfully pursued using the small molecule ABT-737, a mimetic for Bcl-2 and BcL-xL[69]. ABT-737 has shown prominent activity towards various different types of tumour. Recently, ABT-737 was also shown to markedly prolong survival in an intracranial xenograft GBM model[70]. Moreover, ABT-737 synergistically enhanced the activity of sTRAIL as well as standard chemotherapeutic drugs in GBM cells.

Another approach of particular interest in modulating TRAIL-sensitivity is the specific up or down-regulation of microRNAs (miRs). MiRs are small (20-22 nucleotide) non-coding RNAs that degrade or inhibit translation of mRNA by binding to recognition sequences on the mRNA sequence. One miR can modulate a number of genes and as such function as a master regulator. In the case of apoptosis signalling for instance, several miRs have been shown to imprint an apoptosis resistant phenotype on tumour cells. Several miRs have been reported to modulate apoptotic signalling by TRAIL and other TNF-family members. In GBM, a specific miR (miR21) has been reported as highly overexpressed in >90% of tumours analyzed. Interestingly, inhibition of miR21 significantly blocked GBM outgrowth, while co-treatment of anti-miR21 therapy with neural stem cells expressing sTRAIL resulted in synergistic inhibition of tumour growth *in vivo*.

An important consideration to make regarding all of these combinatorial strategies is the possible sensitization of normal cells. For instance, synergistic pro-apoptotic anti-cancer activity upon combination of sTRAIL with proteasome inhibition yielded a therapeutic window in hepatoma cells, but was also associated with enhanced toxicity towards hepatocytes[71]. In addition, hepatocytes were strongly sensitized to Fas upon initial priming with TRAIL[72]. Hepatocytes indeed appear the most TRAIL-sensitive type of cell, with aggregated TRAIL preparations strongly reducing hepatocyte viability[73]. Therefore it is apparent that purely homogenous sTRAIL as well as the rational design of non-toxic combinatorial strategies is required for effective anti-cancer strategy in humans.

Target cell-restricted delivery and optimal activation of TRAIL apoptotic signalling

From a conceptual point of view, the efficacy of sTRAIL is likely to be hampered by several factors, including rapid clearance from the circulation by the kidney. Indeed, sTRAIL has an approximate half-life of only 30 min in primates and a similar pharmacokinetic profile in humans in a phase I clinical trial [32, 74]. Together with the ubiquitous expression of TRAIL receptors in the human body this may severely limit tumour accretion.

Moreover, many tumours express higher levels of TRAILR2 compared to TRAILR1, whereas TRAILR2 signalling is only poorly activated by sTRAIL[75].

We and others have attempted to overcome these drawbacks by fusing sTRAIL to an antibody derivative, such as an antibody fragment. The resultant trimeric molecule will be ~180 kDa and likely has a longer circulation half-life, since renal clearance should be impeded at these higher molecular weights. The antibody targeting domain of the fusion protein will ensure better tumour-accretion and retention (for schematic see figure 4, pg 185) [76-s80]. Importantly, antibody fragment-mediated binding to a cell surface-expressed target antigen converts the sTRAIL into membrane-bound TRAIL that efficiently signals apoptosis via TRAILR1 but also TRAILR2 in a mono- and/or bi/multi-cellular manner[81,82]. In this way also neighbouring tumour cells devoid of target antigen can be effectively eliminated by the so-called bystander effect[83]. The promise of this approach has been shown preclinically in vitro and in vivo for both solid tumours and leukaemia[76,77,78,79].

Of particular interest for GBM is the targeted delivery of sTRAIL to the Epidermal Growth Factor Receptor (EGFR) using EGFR-blocking antibody fragment scFv425. Binding of this blocking antibody fragment to EGFR inhibited EGFR-mitogenic signalling, while the sTRAIL domain at the same time efficiently activated TRAILR-apoptotic signalling (for schematic see figure 5, pg 186)[78]. Obviously this bifurcate strategy of inhibition of tumourigenic EGFR signalling and simultaneous activation of apoptotic signalling is of great appeal for GBM. Moreover, dual EGFR-inhibition by further combination with EGFR tyrosine kinase inhibitor Iressa synergistically enhanced apoptosis by scFv425:sTRAIL. Based on the available data, we further attempted to exploit a reportedly TRAILR1 selective mutant for targeted therapy to EGFR-positive tumour cells. This EGFR-targeted sTRAIL mutant showed a significantly higher activity on ~50% of the cell lines analyzed, whereas it lacked activity towards normal human hepatocytes. However, in our experiments we identified residual binding as well as signalling capacity for TRAILR2[76]. Although the sTRAIL mutant may not be TRAIL-receptor selective, the augmented activity upon targeted delivery to EGFR indicates that the targeted delivery of rationally designed sTRAIL mutants may help to optimize TRAIL-based therapy.

Gene delivery strategies for TRAIL-GBM therapy

As described above, sTRAIL has a rather poor half-life and is likely to be poorly delivered to the tumour. This holds particularly true for GBM cells in the infiltrating zone, where the Blood Brain Barrier still functions and will hamper tumour accumulation of sTRAIL. Several groups have attempted to circumvent these problems by using gene therapeutic approaches. A particularly interesting approach is the transduction of neural stem cells with sTRAIL. Neural stem cells exhibit extensive tropism for GBM and have been shown

to migrate towards outgrowing microsatellites. Thus, secretion of sTRAIL by these cells will ensure GBM localized production. Various preclinical studies have revealed a potent anti-GBM effect of TRAIL-transduced neural stem cells. Of note, combinatorial strategies with these neural stem cells and temozolomide synergistically inhibited GBM outgrowth. In an analogous fashion, the use of human umbilical cord blood-derived mesenchymal stem cells (UCB-MSC) transduced with sTRAIL resulted in prolonged survival of GBM bearing mice. The advantage of these cells over neural stem cells may lie in the ease of isolation and expansion compared to neural stem cells. In addition, ethical problems may be less of an issue for the latter cell type. Next to the use of cell-based strategies, direct TRAIL gene delivery to the tumour using e.g. adenoviruses or Adenovirus associated vectors (AAV) has also resulted in promising preclinical activity *in vivo*.

Delivery strategy for TRAIL-based GBM therapy

An important issue for any GBM-targeted therapeutic strategy is the selective delivery to the vicinity of the tumour. Animal bio-distribution studies with radio-iodinated rhTRAIL (^{125}I-rhTRAIL) have demonstrated that intravenous injection of TRAIL does not yield detectable levels of TRAIL in the brain. Therefore, local delivery strategies such as Convection enhanced delivery (CED) seem more appropriate. CED uses positive pressure infusion to achieve loco-regional delivery of therapeutic agents through an intra-cerebral catheter[84,85]. In animal models, CED can achieve locally high and effective concentrations. By now CED has progressed into phase III clinical studies for immunotoxin delivery [86,87], results of which are likely to yield insight into the feasibility of using CED on a routine basis in GBM patients and its potential applicability for TRAIL-based therapy.

Conclusions and perspectives

The ample preclinical data on GBM cell lines and primary GBM tissue, as well as the notable absence of TRAIL-related toxicity in phase I clinical trials, clearly highlight that TRAIL receptor-targeted strategies hold great appeal for future cancer and more specifically GBM therapy. However, it is also evident from the available literature that GBM is unlikely to be sufficiently responsive to single agent therapy with TRAIL-receptor targeted strategies. Indeed, when taking into account the inherent heterogeneity of GBM it seems most prudent to examine the feasibility of combinatorial strategies that on the one hand sensitize GMB cells to apoptosis, and on the other hand induce apoptosis using TRAIL or agonistic TRAIL-receptor antibodies. As highlighted in this review, TRAIL can be combined with a variety of different conventional and novel therapeutic strategies to yield synergistic pro-apoptotic activity. Of particular appeal in our opinion is the use of dual purpose TRAIL-based molecules, such as the EGFR-targeted TRAIL fusion protein scFv425:sTRAIL. This fusion protein simultaneously blocks EGFR-mitogenic signalling; thereby sensitizing tumour cells to apoptosis, and induces apoptosis via TRAIL-receptor signalling. This fusion protein efficiently activates apoptosis and shows promising *in vivo* activity. Obviously, further rational combination with other therapeutic strategies may help to optimize anti-GBM activity.

An important aspect in considering GBM therapy is the observation that, as in many other types of tumour, a so-called 'stem cell' population can be identified in GBM. These Glioblastoma Stem Cells (GSCs) can re-grow into original glioblastoma in xenograft nude mouse models and express neural stem cell markers, such as CD133. Importantly, GSCs are particularly refractory to radiotherapy and chemotherapy due to e.g. over-expression of multidrug resistance pumps and over-expression of aldehyde dehydrogenase. A recent report identified, in 2 primary patient-derived GSC cultures, that these cells were also refractory to sTRAIL treatment, partly due to selective down-regulation of caspase-8. Whether or not the sTRAIL used in this study was also capable of efficiently activating TRAIL-R2 and whether for instance target cell-restricted activation of TRAIL using scFv:sTRAIL could restore sensitivity of these cells to apoptotic elimination TRAIL is not clear and warrants further investigation. Regardless, this study does serve to illustrate the heterogeneity of GBM, with certain subpopulations that may be (more) refractory to TRAIL-treatment and further illustrates the need for combinatorial therapeutic approaches. Indeed, in a study with the Bcl-2 mimetic ABT-737 the GSC subpopulation of cells was more resistant to treatment than the non-GSC population. This resistance was likely due to over-expression of the anti-apoptotic Bcl-2 family member Mcl-1, already known to confer resistance to ABT-737 in other tumour cell types[88]. Therefore, effective treatment regimes have to include the GSC subpopulation and capitalize on synergistic and complementary activities of the individual reagents.

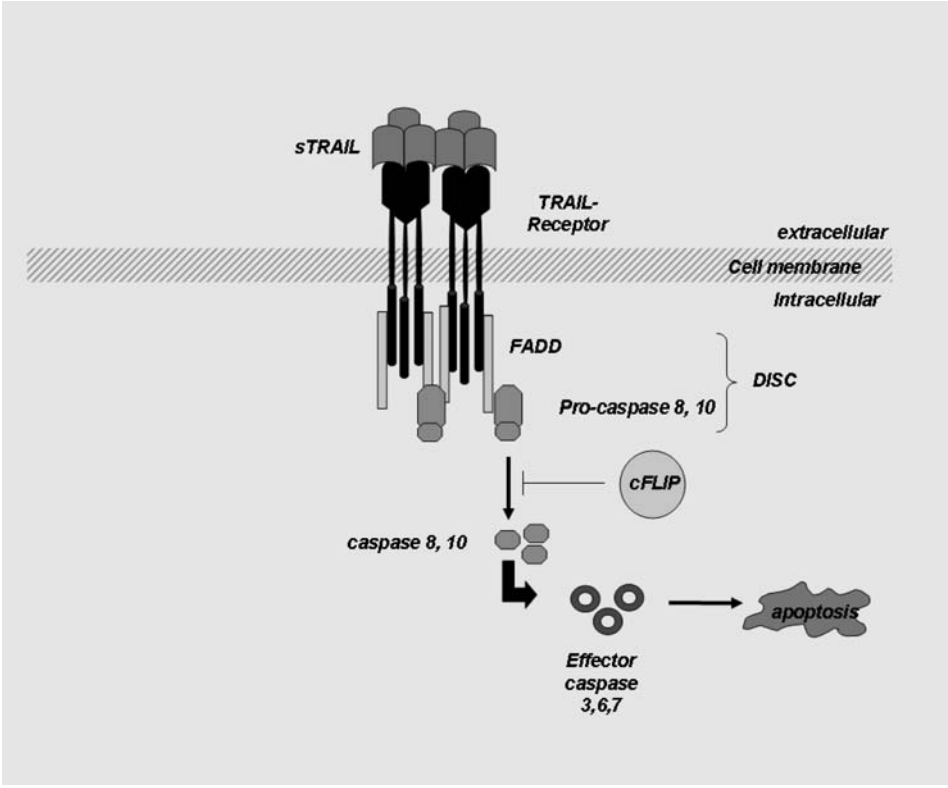
As reported above, the specific modulation of miRs may be of particular interest, since mIR modulation influences the expression of a number of genes and as such can function as a

master regulator. Recent efforts in this field have also helped identify several miR families that are involved in 'stem cell-ness', including let-7 and miR-200. Therefore, rational integration of therapeutic miR modulation with TRAIL (and conventional) therapy may prove an elegant way of shifting the intrinsic cellular balance of normal GBM cells and GBM stem cells towards apoptotic elimination. In a related fashion, the use of small inhibitory RNA to selectively down-regulate an important anti-apoptotic gene, such as cFLIP, may be applied to sensitize GBM for TRAIL-based strategies. The use of siRNA has to date been limited by the question of selective delivery to target cells. However, in a recent seminal paper the use of antibody fragment-targeted anti-HIV siRNA proved successful in curing HIV-infected mice. A similar approach may be adapted to GBM. Indeed, GBM is one of the few cancers reported to express a tumour-specific antigen, the EGFR variant III, for which the MR1-1 antibody fragment is available. Thus, GBM seems an ideal candidate to test the applicability of this novel scFv-siRNA approach in cancer.

Obviously, the application of such rational combinatorial strategies critically depends on the proper identification of specific cancer-related aberrancies in each individual patient/tumour as well as the ability to monitor biological response via e.g. downstream pathway components. Therefore, further development of reliable, cost-effective and high-throughput diagnostic tools will be required to enable the successful application of such patient-tailored therapeutic approaches. Such molecular profiling for GBM is still in its infancy but has gained attraction in recent years with several useful markers available, including EGFRvIII[89].

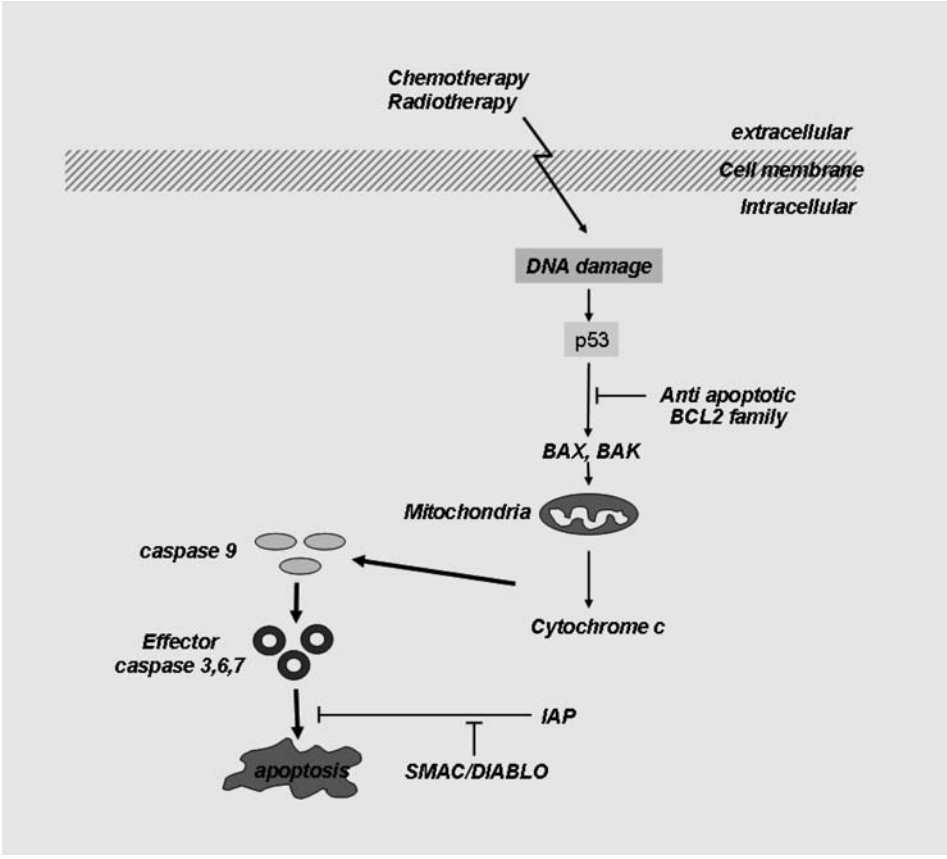
In conclusion, the vast body of evidence from preclinical data indicates that the rational design of combinatorial TRAIL-based approaches with conventional as well as novel therapeutics may ultimately help to combat GBM and improve patient survival for this devastating disease.

Figure 1



The extrinsic apoptosis pathway triggers apoptosis independently of p53 in response to pro-apoptotic ligands, such as Apo2L/TRAIL. These ligands activate specific pro-apoptotic receptors, such as TRAIL-R1 and TRAIL-R2. TRAIL-R1 can induce apoptosis after binding non-cross-linked and cross-linked sTRAIL. TRAIL-R2 can only be activated by cross-linked sTRAIL. Death receptor binding leads to the recruitment of the adaptor FADD and initiator procaspase-8 and 10 to rapidly form the DISC. Procaspase-8 and 10 are cleaved into its activated configuration caspase-8 and 10. Caspase-8 and 10 in turn activate the effector caspase-3, 6 and 7, so triggering apoptosis. cFLIP, cellular FLICE-inhibitory protein; DISC, death-inducing signalling complex; FADD, Fas-associated death domain; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

Figure 2



The intrinsic apoptosis pathway is triggered in response to DNA damage and other types of severe cell stress and involves the release of pro-apoptotic factors from the mitochondria. The p53oncogene protein activates the pro-apoptotic BCL2 family proteins BAX and BAK which leads to the release of cytochrome c. Cytochrome c activates the apoptotic protease caspase 9. Caspase 9, in turn, activates downstream caspases, including caspase 3, 6, and 7, leading to apoptosis. SMAC/DIABLO, directly interacts with inhibitor of apoptosis (IAP) proteins, preventing them from attenuating apoptosis. Anti-apoptotic members of the BCL2 family regulate the mitochondria-initiated caspase activation pathway by preventing the release of cytochrome c (BAK: BCL2 homologous antagonist/killer; BAX: BCL2-associated protein; BCL2: B-cell chronic lymphocytic leukemia/lymphoma 2; IAP, inhibitor of apoptosis protein; SMAC: second mitochondria-derived activator of caspase; DIABLO: direct IAP binding protein with low pI).

References

1. Walker, M. D., Green, S. B., Byar, D. P., Alexander E Jr, Batzdorf, U., Brooks, W. H., Hunt, W. E., MacCarty, C. S., Mahaley, M. S., Jr., Mealey, J., Jr., Owens, G., Ransohoff, J., Robertson, J.T., Shapiro, W. R., Smith, K. R., Jr., Wilson, C. B., and Strike, T. A. Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med 1980; 303: 1323-9
2. Chang, C. H., Horton, J., Schoenfeld, D., Salazer, O., Perez-Tamayo, R., Kramer, S., Weinstein, A., Nelson, J. S., and Tsukada, Y. Comparison of postoperative radiotherapy and combined postoperative radiotherapy and chemotherapy in the multidisciplinary management of malignant gliomas. A joint Radiation Therapy Oncology Group and Eastern Cooperative Oncology Group study. Cancer 1983; 52: 997-1007
3. Fine, H. A., Dear, K. B., Loeffler, J. S., Black, P. M., and Canellos, G. P. Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. Cancer 1993; 71: 2585-97
4. Stewart, L. A. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet 2002; 359: 1011-8
5. Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., and Mirimanoff, R. O. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352: 987-96
6. Hayakawa, Y., Screpanti, V., Yagita, H., Grandien, A., Ljunggren, H. G., Smyth, M. J., and Chambers, B. J. NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy. J Immunol 2004; 172: 123-9
7. Janssen, E. M., Droin, N. M., Lemmens, E. E., Pinkoski, M. J., Bensinger, S. J., Ehst, B. D., Griffith, T. S., Green, D. R., and Schoenberger, S. P. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature 2005; 434: 88-93
8. Smyth, M. J., Takeda, K., Hayakawa, Y., Peschon, J. J., van den Brink, M. R., and Yagita, H. Nature's TRAIL--on a path to cancer immunotherapy. Immunity 2003; 18: 1-6
9. Bouralexis, S., Findlay, D. M., and Evdokiou, A. Death to the bad guys: targeting cancer via Apo2L/TRAIL. Apoptosis 2005; 10: 35-51
10. Roth, W., Isenmann, S., Naumann, U., Kugler, S., Bahr, M., Dichgans, J., Ashkenazi, A., and Weller, M. Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem Biophys Res Commun 1999; 265: 479-83
11. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and . Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 1995; 3: 673-82
12. Mariani, S. M. and Krammer, P. H. Differential regulation of TRAIL and CD95 ligand in trans formed cells of the T and B lymphocyte lineage. Eur J Immunol 1998; 28: 973-82
13. Monleon, I., Martinez-Lorenzo, M. J., Monteagudo, L., Lasierra, P., Taules, M., Iturralde, M., Pineiro, A., Larrad, L., Alava, M. A., Naval, J., and Anel, A. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. J Immunol 2001; 167: 6736-44
14. Breckenridge, D. and Xue, D. Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. Current Opinion in Cell Biology 2004; 16: 647-52
15. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol 1997; 7: 1003-6

16. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997; 7: 813-20
17. Spierings, D. C., de Vries, E. G., Vellenga, E., van den Heuvel, F. A., Koornstra, J. J., Wesseling, J., Hollema, H., and de Jong, S. Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem 2004; 52: 821-31
18. Kumamoto, H. and Ooya, K. Expression of tumor necrosis factor alpha, TNF-related apoptosis-inducing ligand, and their associated molecules in ameloblastomas. J Oral Pathol Med 2005; 34: 287-94
19. Melloni, E., Secchiero, P., Celeghini, C., Campioni, D., Grill, V., Guidotti, L., and Zauli, G. Functional expression of TRAIL and TRAIL-R2 during human megakaryocytic development. J Cell Physiol 2005; 204: 975-82
20. Secchiero, P., Melloni, E., Heikinheimo, M., Mannisto, S., Di Pietro, R., Iacone, A., and Zauli, G. TRAIL regulates normal erythroid maturation through an ERK-dependent pathway. Blood 2004; 103: 517-22
21. Strater, J., Hinz, U., Walczak, H., Mechtersheimer, G., Koretz, K., Herfarth, C., Moller, P., and Lehnert, T. Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. Clin Cancer Res 2002; 8: 3734-40
22. Daniels, R. A., Turley, H., Kimberley, F. C., Liu, X. S., Mongkolsapaya, J., Ch'En, P., Xu, X. N., Jin, B. Q., Pezzella, F., and Screaton, G. R. Expression of TRAIL and TRAIL receptors in normal and malignant tissues. Cell Res 2005; 15: 430-8
23. Frank, S., Kohler, U., Schackert, G., and Schackert, H. K. Expression of TRAIL and its receptors in human brain tumors. Biochem Biophys Res Commun 1999; 257: 454-9
24. Dorr, J., Bechmann, I., Waiczies, S., Aktas, O., Walczak, H., Krammer, P. H., Nitsch, R., and Zipp, F. Lack of tumor necrosis factor-related apoptosis-inducing ligand but presence of its receptors in the human brain. J Neurosci 2002; 22: RC209
25. Arts, H. J., de Jong, S., Hollema, H., ten Hoor, K., van der Zee, A. G., and de Vries, E. G. Chemotherapy induces death receptor 5 in epithelial ovarian carcinoma. Gynecol Oncol 2004; 92: 794-800
26. Stander, S. and Schwarz, T. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is expressed in normal skin and cutaneous inflammatory diseases, but not in chronically UV-exposed skin and non-melanoma skin cancer. Am J Dermatopathol 2005; 27: 116-21
27. McCarthy, M. M., DiVito, K. A., Sznol, M., Kovacs, D., Halaban, R., Berger, A. J., Flaherty, K. T., Camp, R. L., Lazova, R., Rimm, D. L., and Kluger, H. M. Expression of tumor necrosis factor--related apoptosis-inducing ligand receptors 1 and 2 in melanoma. Clin Cancer Res 2006; 12: 3856-63
28. van Geelen, C. M., Westra, J. L., de Vries, E. G., Boersma-van Ek, W., Zwart, N., Hollema, H., Boezen, H. M., Mulder, N. H., Plukker, J. T., de Jong, S., Kleibeuker, J. H., and Koornstra, J. J. Prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand and its receptors in adjuvantly treated stage III colon cancer patients. J Clin Oncol 2006; 24: 4998-5004
29. Kuijlen, J. M., Mooij, J. J., Platteel, I., Hoving, E. W., van der Graaf, W. T., Span, M. M., Hollema, H., and den Dunnen, W. F. TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. J Neurooncol 2006; .78: 161-71
30. Knight, M. J., Riffkin, C. D., Muscat, A. M., Ashley, D. M., and Hawkins, C. J. Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. Oncogene 2001; 20: 5789-98
31. Hetschko, H., Voss, V., Horn, S., Seifert, V., Prehn, J. H., and Kogel, D. Pharmacological inhibition of Bcl-2 family members reactivates TRAIL-induced apoptosis in malignant glioma. J Neurooncol 2008; 86: 265-72
32. Ashkenazi, Avi, Holland, Pamela, and Eckhardt, S. Gail. Ligand-Based Targeting of Apoptosis

in Cancer: The Potential of Recombinant Human Apoptosis Ligand 2/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhApo2L/TRAIL). J Clin Oncol 2008; 26: 3621-30
33. Hotte, S. J., Hirte, H. W., Chen, E. X., Siu, L. L., Le, L. H., Corey, A., Iacobucci, A., Maclean, M., Lo, L., Fox, N. L., and Oza, A. M. A Phase 1 Study of Mapatumumab (Fully Human Monoclonal Antibody to TRAIL-R1) in Patients with Advanced Solid Malignancies. Clin Cancer Res 2008; 14: 3450-5
34. Marini, P. Drug evaluation: lexatumumab, an intravenous human agonistic mAb targeting TRAIL receptor 2. Curr Opin Mol Ther 2006; 8: 539-46
35. Ashkenazi, A. Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev 2008; 19: 325-31
36. Tolcher, A. W., Mita, M., Meropol, N. J., von Mehren, M., Patnaik, A., Padavic, K., Hill, M., Mays, T., McCoy, T., Fox, N. L., Halpern, W., Corey, A., and Cohen, R. B. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007; 25: 1390-5
37. Greco, F. A., Bonomi, P., Crawford, J., Kelly, K., Oh, Y., Halpern, W., Lo, L., Gallant, G., and Klein, J. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. Lung Cancer 2008; .:
38. Duiker, E. W., Mom, C. H., de Jong, S., Willemse, P. H., Gietema, J. A., van der Zee, A. G., and de Vries, E. G. The clinical trail of TRAIL. Eur J Cancer 2006; 42: 2233-40
39. C.H.Mom, I.A.Sloots, S.de Jong, J.A.Gietema, E.G.E.de Vries, and S.Sleijfer. TRAIL modulators. 2007; 207
40. Kelley, Robert F., Totpal, Klara, Lindstrom, Stephanie H., Mathieu, Mary, Billeci, Karen, DeForge, Laura, Pai, Roger, Hymowitz, Sarah G., and Ashkenazi, Avi. Receptor-selective Mutants of Apoptosis-inducing Ligand 2/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Reveal a Greater Contribution of Death Receptor (DR) 5 than DR4 to Apoptosis Signaling. J Biol Chem 2005; 280: 2205-12
41. MacFarlane, M., Kohlhaas, S., Sutcliffe, M., Dyer, M., and Cohen, G. TRAIL Receptor-Selective Mutants Signal to Apoptosis via TRAIL-R1 in Primary Lymphoid Malignancies. Cancer Res 2005; 65: 11265-70
42. Duiker, E. W., de Vries, E. G., Mahalingam, D., Meersma, G. J., Boersma-van Ek, W., Hollema, H., Lub-de Hooge, M. N., van Dam, G. M., Cool, R. H., Quax, W. J., Samali, A., van der Zee, A. G., and de Jong, S. Enhanced antitumor efficacy of a DR5-specific TRAIL variant over recombinant human TRAIL in a bioluminescent ovarian cancer xenograft model. Clin Cancer Res 2009; 15: 2048-57
43. Reis, C. R., van der Sloot, A. M., Szegezdi, E., Natoni, A., Tur, V., Cool, R. H., Samali, A., Serrano, L., and Quax, W. J. Enhancement of Antitumor Properties of rhTRAIL by Affinity Increase toward Its Death Receptors (dagger). Biochemistry 2009; 48: 2180-91
44. Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. Inhibition of death receptor signals by cellular FLIP. Nature 1997; 388: 190-5
45. Wang, J., Lobito, A. A., Shen, F., Hornung, F., Winoto, A., and Lenardo, M. J. Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. Eur J Immunol 2000; 30: 155-63
46. Condorelli, G., Vigliotta, G., Cafieri, A., Trencia, A., Andalo, P., Oriente, F., Miele, C., Caruso, M., Formisano, P., and Beguinot, F. PED/PEA-15: an anti-apoptotic molecule that regulates FAS/TNFR1-induced apoptosis. Oncogene 1999; 18: 4409-15
47. Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M., and Sorger, P. K. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature 2009; 459: 428-32
48. Goy, A. New directions in the treatment of mantle cell lymphoma: an overview.

Clin Lymphoma Myeloma 2006; 7 Suppl 1: S24-S32
49. Maddipatla, Sreeram, Hernandez-Ilizaliturri, Francisco J., Knight, Joy, and Czuczman, Myron S. Augmented Antitumor Activity against B-Cell Lymphoma by a Combination of Monoclonal Antibodies Targeting TRAIL-R1 and CD20. Clin Cancer Res 2007; 13: 4556-64
50. Daniel, Dylan, Yang, Becky, Lawrence, David A., Totpal, Klara, Balter, Inessa, Lee, Wyne P., Gogineni, Alvin, Cole, Mary J., Yee, Sharon Fong, Ross, Sarajane, and Ashkenazi, Avi. Cooperation of the proapoptotic receptor agonist rhApo2L/TRAIL with the CD20 antibody rituximab against non-Hodgkin lymphoma xenografts. Blood 2007; 110: 4037-46
51. Ciusani, E., Croci, D., Gelati, M., Calatozzolo, C., Sciacca, F., Fumagalli, L., Balzarotti, M., Fariselli, L., Boiardi, A., and Salmaggi, A. In vitro effects of topotecan and ionizing radiation on TRAIL/Apo2L-mediated apoptosis in malignant glioma. J Neurooncol 2005; 71: 19-25
52. Zhou, Q., Fukushima, P., DeGraff, W., Mitchell, J. B., Stetler, Stevenson M., Ashkenazi, A., and Steeg, P. S. Radiation and the Apo2L/TRAIL apoptotic pathway preferentially inhibit the colonization of premalignant human breast cells overexpressing cyclin D1. Cancer Res 2000; 60: 2611-5
53. Di Pietro, R., Secchiero, P., Rana, R., Gibellini, D., Visani, G., Bemis, K., Zamai, L., Miscia, S., and Zauli, G. Ionizing radiation sensitizes erythroleukemic cells but not normal erythroblasts to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)--mediated cytotoxicity by selective up-regulation of TRAIL-R1. Blood 2001; 97: 2596-603
54. Ramp, U., Caliskan, E., Mahotka, C., Krieg, A., Heikaus, S., Gabbert, H. E., and Gerharz, C. D. Apoptosis induction in renal cell carcinoma by TRAIL and gamma-radiation is impaired by deficient caspase-9 cleavage. Br J Cancer 2003; 88: 1800-7
55. Kim, M. R., Lee, J. Y., Park, M. T., Chun, Y. J., Jang, Y. J., Kang, C. M., Kim, H. S., Cho, C. K., Lee, Y. S., Jeong, H. Y., and Lee, S. J. Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. FEBS Lett 2001; 505: 179-84
56. Chinnaiyan, A. M., Prasad, U., Shankar, S., Hamstra, D. A., Shanaiah, M., Chenevert, T. L., Ross, B. D., and Rehemtulla, A. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 2000; 97: 1754-9
57. Gong, B. and Almasan, A. Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. Cancer Res 2000; 60: 5754-60
58. Nagane, M., Cavenee, W. K., and Shiokawa, Y. Synergistic cytotoxicity through the activation of multiple apoptosis pathways in human glioma cells induced by combined treatment with ionizing radiation and tumor necrosis factor-related apoptosis-inducing ligand. J Neurosurg 2007; 106: 407-16
59. Nagane, M., Pan, G., Weddle, J. J., Dixit, V. M., Cavenee, W. K., and Huang, H. J. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res 2000; 60: 847-53
60. Song, J. H., Song, D. K., Pyrzynska, B., Petruk, K. C., Van Meir, E. G., and Hao, C. TRAIL triggers apoptosis in human malignant glioma cells through extrinsic and intrinsic pathways. Brain Pathol 2003; 13: 539-53
61. Rohn, T. A., Wagenknecht, B., Roth, W., Naumann, U., Gulbins, E., Krammer, P. H., Walczak, H., and Weller, M. CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. Oncogene 2001; 20: 4128-37
62. Saito, R., Bringas, J. R., Panner, A., Tamas, M., Pieper, R. O., Berger, M. S., and Bankiewicz, K. S. Convection-enhanced delivery of tumor necrosis factor-related apoptosis-inducing ligand with systemic administration of temozolomide prolongs survival in an intracranial glioblastoma xenograft model. Cancer Res 2004; 64: 6858-62

63. Koschny, R., Holland, H., Sykora, J., Haas, T. L., Sprick, M. R., Ganten, T. M., Krupp, W., Bauer, M., Ahnert, P., Meixensberger, J., and Walczak, H. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Clin Cancer Res 2007; 13: 3403-12
64. Mani, A. and Gelmann, E. The Ubiquitin-Proteasome Pathway and Its Role in Cancer. J Clin Oncol 2005; 23: 4776-89
65. Fernandez, Y., Verhaegen, M., Miller, T., Rush, J., Steiner, P., Opipari, A., Lowe, S., and Soengas, M. Differential Regulation of Noxa in Normal Melanocytes and Melanoma Cells by Proteasome Inhibition: Therapeutic Implications. Cancer Res 2005; 65: 6294-304
66. Brooks, A. L. A. N., Ramirez, T. E. R. E., Toh, U. H. I., Onksen, J. E. N. N., Elliott, P. E. T. E., Murphy, W. I. L. L., and Sayers, T. H. O. M. The Proteasome Inhibitor Bortezomib (Velcade) Sensitizes Some Human Tumor Cells to Apo2L/TRAIL-Mediated Apoptosis. Ann NY Acad Sci 2005; 1059: 160-7
67. Panner, A., Parsa, A. T., and Pieper, R. O. Use of APO2L/TRAIL with mTOR inhibitors in the treatment of glioblastoma multiforme. Expert Rev Anticancer Ther 2006; 6: 1313-22
68. Tamm, I. AEG-35156, an antisense oligonucleotide against X-linked inhibitor of apoptosis for the potential treatment of cancer. Curr Opin Investig Drugs 2008; 9: 638-46
69. Oltersdorf, T., Elmore, S., Shoemaker, A., Armstrong, R., Augeri, D., Belli, B., Bruncko, M., Deckwerth, T., Dinges, J., Hajduk, P., Joseph, M., Kitada, S., Korsmeyer, S., Kunzer, A., Letai, A., Li, C., Mitten, M., Nettesheim, D., Ng, S., Nimmer, P., O'Connor, J., Oleksijew, A., Petros, A., Reed, J., Shen, W., Tahir, S., Thompson, C., Tomaselli, K., Wang, B., Wendt, M., Zhang, H., Fesik, S., and Rosenberg, S. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005; 435: 677-81
70. Tagscherer, K. E., Fassl, A., Campos, B., Farhadi, M., Kraemer, A., Bock, B. C., Macher-Goeppinger, S., Radlwimmer, B., Wiestler, O. D., Herold-Mende, C., and Roth, W. Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins. Oncogene 2008; 27: 6646-56
71. Koschny, R., Ganten, T. M., Sykora, J., Haas, T. L., Sprick, M. R., Kolb, A., Stremmel, W., and Walczak, H. TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. Hepatology 2007; 45: 649-58
72. Corazza, N., Jakob, S., Schaer, C., Frese, S., Keogh, A., Stroka, D., Kassahn, D., Torgler, R., Mueller, C., Schneider, P., and Brunner, T. TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. J Clin Invest 2006; 116: 2493-9
73. Gores, G. J. and Kaufmann, S. H. Is TRAIL hepatotoxic? Hepatology 2001; 34: 3-6
74. Kelley, S. K., Harris, L. A., Xie, D., DeForge, L., Totpal, K., Bussiere, J., and Fox, J. A. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. The Journal Of Pharmacology And Experimental Therapeutics 2001; 299: 31-8
75. Muhlenbeck, F., Schneider, P., Bodmer, J. L., Schwenzer, R., Hauser, A., Schubert, G., Scheurich, P., Moosmayer, D., Tschopp, J., and Wajant, H. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J Biol Chem 2000; 275: 32208-13
76. Bremer, E., de, Bruyn M., Samplonius, D. F., Bijma, T., ten, Cate B., de Leij, L. F., and Helfrich, W. Targeted delivery of a designed sTRAIL mutant results in superior apoptotic activity towards EGFR-positive tumor cells. J Mol Med 2008; 86: 909-24
77. Bremer, E., Samplonius, D. F., Peipp, M., van, Genne L., Kroesen, B. J., Fey, G. H., Gramatzki, M., de Leij, L. F., and Helfrich, W. Target cell-restricted apoptosis induction of acute leukemic T cells by a recombinant tumor necrosis factor-related apoptosis-inducing ligand fusion protein with specificity for human CD7. Cancer Res 2005; 65: 3380-8

78. Bremer, E., Samplonius, D. F., van Genne, L., Dijkstra, M. H., Kroesen, B. J., de Leij, L. F., and Helfrich, W. Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. J Biol Chem 2005; 280: 10025-33
79. Stieglmaier, J., Bremer, E., Kellner, C., Liebig, T. M., ten, Cate B., Peipp, M., Schulze-Koops, H., Pfeiffer, M., Buhring, H. J., Greil, J., Oduncu, F., Emmerich, B., Fey, G. H., and Helfrich, W. Selective induction of apoptosis in leukemic B-lymphoid cells by a CD19-specific TRAIL fusion protein. Cancer Immunol Immunother 2008; 57: 233-46
80. Bremer, Edwin, van Dam, Gooitzen M., de Bruyn, Marco, van Riezen, Manon, Dijkstra, Marike, Kamps, Gera, Helfrich, Wijnand, and Haisma, Hidde. Potent Systemic Anticancer Activity of Adenovirally Expressed EGFR-Selective TRAIL Fusion Protein. Mol Ther 2008;
81. Wajant, H., Moosmayer, D., Wuest, T., Bartke, T., Gerlach, E., Schonherr, U., Peters, N., Scheurich, P., and Pfizenmaier, K. Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 2001; 20: 4101-6
82. Bremer, E., Kuijlen, J., Samplonius, D., Walczak, H., de Leij, L., and Helfrich, W. Target cell-restricted and -enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. Int J Cancer 2004; 109: 281-90
83. Bremer, E., Samplonius, D., Kroesen, B. J., van, Genne L., de, Leij L., and Helfrich, W. Exceptionally potent anti-tumor bystander activity of an scFv:sTRAIL fusion protein with specificity for EGP2 toward target antigen-negative tumor cells. Neoplasia 2004; 6: 636-45
84. Laske, D. W., Ilercil, O., Akbasak, A., Youle, R. J., and Oldfield, E. H. Efficacy of direct intratumoral therapy with targeted protein toxins for solid human gliomas in nude mice. J Neurosurg 1994; 80: 520-6
85. Laske, D. W., Morrison, P. F., Lieberman, D. M., Corthesy, M. E., Reynolds, J. C., Stewart-Henney, P. A., Koong, S. S., Cummins, A., Paik, C. H., and Oldfield, E. H. Chronic interstitial infusion of protein to primate brain: determination of drug distribution and clearance with single-photon emission computerized tomography imaging. J Neurosurg 1997; 87: 586-94
86. Kioi, M., Husain, S. R., Croteau, D., Kunwar, S., and Puri, R. K. Convection-enhanced delivery of interleukin-13 receptor-directed cytotoxin for malignant glioma therapy. Technol Cancer Res Treat 2006; 5: 239-50
87. Vandergrift, W. A., Patel, S. J., Nicholas, J. S., and Varma, A. K. Convection-enhanced delivery of immunotoxins and radioisotopes for treatment of malignant gliomas. Neurosurg Focus 2006; 20: E13
88. Fleischer, B., Schulze-Bergkamen, H., Schuchmann, M., Weber, A., Biesterfeld, S., Muller, M., Krammer, P. H., and Galle, P. R. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma 3. Int J Oncol 2006; 28: 25-32
89. Mladkova, N. and Chakravarti, A. Molecular profiling in glioblastoma: prelude to personalized treatment. Curr Oncol Rep 2009; 11: 53-61

Chapter 3

TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme

Jos M. A. Kuijlen[1], Jan Jakob A. Mooij[1], Inge Platteel[2], Eelco W. Hoving[1], Winette T.A. van der Graaf[3], Mark M Span[4], Harry Hollema[2], Wilfred F.A. den Dunnen[2]

[1]Department of Neurosurgery
[2]Department of Pathology and Laboratory Medicine
[3]Department of Internal Medicine
[4]Department of Medical Technology Assessment
University Medical Center Groningen, The Netherlands

J Neurooncol 2006;78(2):161-71

Abstract

Purpose:
In order to improve the survival of patients with a glioblastoma multiforme tumor (GBM), new therapeutic strategies must be developed. The use of a death inducing ligand such as TRAIL (TNF Related Apoptosis Inducing Ligand) seems a promising innovative therapy. The aim of this study was to quantify the expression of the death regulating receptors TRAIL-R1, TRAIL-R2 and TRAIL on primary GBM specimens and to correlate this expression with survival.

Experimental design:
Expression of TRAIL and TRAIL-receptors was assessed by immunohistochemistry, both quantitatively (% of positive tumor cells) and semi-quantitatively (staining intensity) within both the perinecrotic and intermediate tumor zones of primary GBM specimens. RT-PCR of GBM tissue was performed to show expression of TRAIL receptor mRNA.

Results:
Immunohistochemistry showed a slight diffuse intracytoplasmic and a stronger membranous staining for TRAIL and TRAIL receptors in tumor cells. Semi-quantitative expression of TRAIL showed a significantly higher expression of TRAIL in the perinecrotic zone than in the intermediate zone of the tumor (p=0.0001). TRAIL-R2 expression was significantly higher expressed than TRAIL-R1 (p=0.005). The antigenic load of TRAIL-R2 was positively correlated with survival (p=0.02). Multivariate analysis of TRAIL-R1 within the study group (n=62) showed that age, gender, staining intensity, antigenic load, % of TRAIL-R1 expression, were not statistically correlated with survival however radiotherapy was significantly correlated (multivariate analysis: age: p=0,15; gender: p=0.64; staining intensity: p=0.17; antigenic load: p= 0.056; % of TRAIL-R1 expression: p=0.058; radiotherapy: p=0.0001). Subgroup analysis of patients who had received radiotherapy (n=47) showed a significant association of % of TRAIL-R1 expression and the antigenic load of TRAIL-R1 with survival (multivariate analysis: p=0.036 respectively p=0.023).

Multivariate analysis of TRAIL-R2 staining intensity and antigenic load, within the study group (p=0.004 resp. p=0.03) and the subgroup (p=0.002 resp. p=0.004), showed a significant association with survival. RT-PCR analysis detected a negative relation between the amount of TRAIL-R1 mRNA and the WHO grade of astrocytic tumors (p=0.03).

Conclusions:
TRAIL-R1 and TRAIL-R2 expression on tumor cells are independent prognostic factors for survival in patients with a glioblastoma multiforme. Both receptors could be targets for TRAIL therapy. As TRAIL-R2 is more expressed, in comparison with TRAIL-R1, on GBM tumor cells, TRAIL-R2 seems to be of more importance as a target for future TRAIL therapy than TRAIL-R1.

Introduction

Neurosurgical debulking of a glioblastoma multiforme tumor followed by localized radiation of the cranium up to 60 Gy is still the standard therapeutic regimen for patients with this highly malignant primary brain tumor. The overall median survival after this convential treatment lies between 9 and 12 months [1]. Radiotherapy has proven to prolong overall median survival for several months. Adjuvant chemotherapy has been investigated in various trials since the 1970's with variable outcome. Although the recently presented Phase III study on Temozolomide (EORTC 26981) showed a statistically significant (2-3 months) prolonged survival for patients with a newly diagnosed GBM [2], this is still a minor gain. Therefore it is worthwhile to develop new therapeutic strategies for patients with a GBM.

One such strategy could be the use of apoptosis inducing ligands to eliminate tumor cells. TRAIL (TNF Related Apoptosis Inducing Ligand) is a promising death inducing ligand as this ligand induces apoptosis by ligation to its cognate cell surface receptors (TRAIL-R1 and R2) [3]. TRAIL can be present as membranous bound (memTRAIL) but can also be proteolytically cleaved from the membrane to form a soluble ligand (sTRAIL) [4]. The soluble form of TRAIL can be used for therapeutic options. Interestingly, soluble TRAIL induces apoptosis in tumor cells, and not in normal tissue [3]. Other data available showing possible side effect of sTRAIL on normal tissues although these might be related to dose or the modified form of sTRAIL used in these experiments [5-7].

TRAIL receptor expression on glioma cell lines has been frequently studied [8, 9-11], however only one study [12] addressed the expression of TRAIL receptors in primary tumor tissue.

The aim of this study was to quantify the expression of the death receptors TRAIL-R1, TRAIL-R2 and the membranous TRAIL, on primary GBM tumor specimens, with immunohistochemistry and RT-PCR. Receptor expression results were correlated with survival.

Material and methods

Patients

A total of 132 patients were diagnosed with a primary glioblastoma multiforme, according to the WHO criteria [13], in our hospital between January 1998 and January 2003. Exclusion criteria for this study were; stereotactic biopsy (57 patients), secondary GBM (10) and mixed tumors containing zones with sarcomatous or oligodendroglial differentiation (3). Finally, tumor tissue of 62 patients was included in this study.

Baseline characteristics

Several baseline patient characteristics were evaluated: gender, age at diagnosis, interval between start of symptoms and operation, postoperative radiotherapy and survival period. “Age at diagnosis” is defined as the age of the patient at the date of the first MRI/CT on which the tumor was diagnosed. The “period between start of symptoms and operation” is defined as the time between the manifestation of the first clinical symptoms, as registered by the referring neurologist, and the operation day. The survival period was calculated as the period from date of surgery until death.

Radiographic parameters

Pre-operative tumor size was measured on a gadolineum enhanced T1 weighted MRI, or if MRI scans were not available, on a contrast enhancing CT scan. Because we did not apply volumetric computer assisted measurements on the pre-operative scans we calculated the tumor volume by assuming that it was a sphere. The length and width of the tumor on the slice with the largest defined mass lesion was summed up and divided by 4 and this outcome was used as the radius.

Immunohistochemistry (IHC)

Paraffin embedded tumor tissue samples from patients with a GBM after debulking, were used. All the specimens were reviewed by the authors WFAdD and HH. Only patient samples with sufficient tissue that met all four WHO criteria [13] of a glioblastoma were used in this study. Tumor tissue from other types of primary brain tumors and normal brain tissue obtained from autopsies were used as control.
Formalin-fixed, paraffin-embed¬ded sections (4 μm) were cleared in xylene and rehydrated in a graded alcohol series. For TRAIL-R2 staining, antigen retrieval was performed by adding slides to a 10 mM citric acid monohydrate (Merck, Darmstadt, Germany) solu-

tion in demineralized water, pH=6.0, and subsequent microwave treatment at 100oC for 8 min at 700 W. Endogeneous peroxidase activity was blocked by treatment of slides with 1% peroxide in phosphate buffered saline solution (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 0.15 mM KH2PO_4, pH 7.8) for 30 min. Slides were then washed twice with PBS and pre-incubated for 15 min with avidin and biotin blocking reagent (Vector Laboratories, Burlingame, CA). Slides were then stained for 60 min with TRAIL antibody (TRAIL (K-18), cat nr: sc-6079 Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1: 25, TRAIL-R2 antibody (TRAIL-R2 (DR5) (Ab-1); cat nr: PC392 Oncogene, Cambridge, MA) diluted 1:100, TRAIL-R1 antibody (DR4 (C-20), cat nr: sc-6823, Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1:100. All antibodies were diluted in PBS, 1% BSA. For the negative controls, no antibody was added to the PBS or the primary antibody was replaced by normal goat IgG (TRAIL, TRAIL-R1) or normal rabbit IgG (TRAIL-R2). Positive controls were tumor tissue samples, found on previous occasions to stain positive and first trimester placenta tissue for TRAIL and kidney tissue for TRAIL-R1 and TRAIL-R2.
After washes in PBS, the sections were exposed to the secondary biotinylated rabbit anti goat IgG diluted 1:300 (TRAIL, TRAIL-R1) or swine anti rabbit diluted 1:300 (TRAIL-R2) for 30 min, followed by the amplification system streptavidin-biotin complex. Secondary antibodies were diluted in PBS with 1% BSA and 1%AB serum. The slides were then treated with DAB and H_2O_2 for 10 min and counterstained with hematoxylin.

Preparation of RNA

Snap-frozen sections (10×10µm), of 19 primary GBM tissue samples, were cut and placed in a tube containing 300 µl lysisbuffer with 2.1 µl Beta-ME. For the RNA-isolation a RNA RT-PCR Miniprep kit (Stratagene) was used. RNA was stored at minus 20 degrees Celsius. Synthesis of cDNA was performed by using Random primers Hexamers. In a PCR-tube 10 µl RNA solution, 1 µl Random primers Hexamers (300 ng) and 1 µl 10 mM dNTP-mix were incubated by 65°C for 5 min. Directly after this the mix was put on ice. Then 4 µl 5× First-Strand buffer, 2 µl 0,1 M DTT and 1 µl RNaseOUT Recombinant Ribonuclease Inhibitor (40 units/µl, Invitrogen) were added. The mixer was incubated at 25 °C for 10 min and thereafter for 2 min at 42 °C before adding 1 µl Superscript II (200 units, Invitrogen). Then the mixer was incubated for 50 min. by 42 °C and for inactivating of the reaction incubated by 70 °C for 15 min. The cDNA was stored at -20°C.

RT-PCR

For the RT-PCR 2 primer sets were designed by using the sequence from PubMed (NM-003842, NM-147187 and NM-003844) and Primer design 3. Polymerase chain reaction for TRAIL R1 was performed using the forward primer 5'-AGAGAGAAGTCCCTGCACCA-'3

and reversed 5'-GTCACTCCAGGGCGTACAAT-'3 and for TRAIL R2 forward 5'-GATGGTCAAGGTCGGTGATT-'3 and reversed 5'-TACGGCTGCAACTGTGACTC-'3. One µl of each cDNA was amplified using PCR Master Mix (Amersham Biosciences) under the following PCR conditions; 5 min. 94 °C, 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 90 s, followed by 72°C for 7 min. The PCR-products were detected on a 1% agarose gel with Ethidiumbromide and analyzed with the geldoc 1000 (Biorad) with the household gene GAPDH as reference. The RT-PCR data from the GBM samples (n=19) was compared to RT-PCR data from other primary astrocytic brain tumors (pilocytic astrocytoma (WHO 1) n=3; diffuse astrocytoma (WHO 2) n=6; anaplastic astrocytoma (WHO 3) n=3; secondary GBM (WHO 4)(n=3) and normal brain tissue (n=4). Splice variants of TRAIL-R2 were also analyzed (TRICK 2a and TRICK 2b).

Method of histological evaluation

All specimens were evaluated by the investigators (WFdD and HH) who were blinded for the patient data. Firstly, a semi-quantitative score was used to define the staining pattern Staining patterns were recorded in the perinecrotic zone and intermediate zone. The perinecrotic zone was defined as the zone immediately surrounding a zone of necrosis. This zone was confirmed with VEGF-A and HIF-1-alpha expression (data not shown) [14-16]. In this relatively hypoxic zone, intracellular processes in tumor cells will differ from tumor cells in the intermediate zone, which is less hypoxic. The intermediate zone was defined as the region between the perinecrotic zone and the zone of invasion of cells into the normal brain tissue. As infiltrating tumor cells originate from the intermediate zone, this zone is the most important zone to evaluate histochemically . The semi-quantitative staining score is defined as a four point scale. No staining was scored as 0, low staining as 1, moderate as 2 and high intensity staining as 3. Secondly, within the intermediate zone, 1 high power field with 400 to 1200 cells was counted for staining of TRAIL or its TRAIL receptors. The percentage of positive cells (% TRAIL-R1 or R2) was calculated. Thirdly, within the intermediate zone the "antigenic load" (AGL) of the tumor was calculated. The antigenic load is the product of the percentage of positive cells and the semi-quantitative staining score. In this way both parameters are incorporated in a single value and this value, as we think, presents a more balanced outcome regarding TRAIL receptor presence in tumor tissue. The antigenic load varies from 0 to 300.

Statistical methods

Statistical comparisons were made with a Student's t-test if data had a Gaussian distribution and a Mann-Whitney U test was used when data was not normally distributed. The non-parametric Spearman correlation test was used to compute correlation. A Kruskal-Wallis test was used to analyze multiple group comparisons. Kaplan-Meier

estimates were used to compute survival. Semiquantitative expression of TRAIL-R1 and TRAIL-R2 (staining intensity and AGL) was dichotomized in order to create larger groups with more power for analyses. No staining (0) and low staining (1) were grouped together into a new category called “staining 0/1”and moderate (2) and high staining (3) were grouped into the category “staining 2/3”. The cut-off point for the antigenic load was set a 20 for TRAIL-R1 and at 40 for TRAIL-R2. These cut-off points were chosen because they represent the median expression for both receptors.

Univariate and multivariate Hazard Ratios and the 95% CI’s were computed using the Cox proportional hazard analyses (SPSS 12). Proportional hazard analyses were done in the total study group and in a group of patients consisting of patients who had received radiotherapy. Within the text this group is called the subgroup. Differences found were significant at the p-level smaller than 0.05. All p-values reported are two-tailed.

Results

Patient characteristics

All patients had a Karnofsky Performance Score (KPS) of 70 or above, before operation. The mean age at diagnosis of the overall group was 59 years (95% CI: 56-62) with a male preponderance (69 %). Mean pre-operative tumor volume was 43 cm^3 (95 % CI 36- 49 cm3). Seventy-six percent of the patients received radiotherapy. The remaining patients (24%) did not receive radiotherapy because of rapid deterioration after surgery, which consequently led to a KPS beneath 70 and radiotherapy was abrogated. None of the patients within this study received any form of chemotherapy because in the study period, in the Netherlands, chemotherapy did not belong to the standard treatment of patients with a GBM. The mean survival of the total group (Figure 1) was 259 days (95% CI: 214-304). Age and gender were not associated with survival (Table 3) in contrast to radiotherapy, which was an independent factor for survival (Table 3).

Immunohistochemical evaluation of GBM tissue samples

In general, immunohistochemistry (IHC) for TRAIL, TRAIL-R1 and TRAIL-R2 displayed diffuse but slight background staining despite avidin-biotin blocking. However, staining of the astrocytes could be clearly distinguished from the background staining. The IHC for TRAIL (Figure 2A, pg 187), TRAIL-R1 (Figure 2B, pg 187) and TRAIL-R2 (Figure 2C, pg 187) showed a diffuse cytoplasmic staining together with a more intense membranous staining.

TRAIL expression

The semi-quantitative assessment showed a significantly stronger TRAIL expression in the perinecrotic zone when compared with the intermediate zone (1,53 vs. 0,90; $p < 0.0001$).

Quantitative measurement showed that 13 % (95% CI: 8-17) of the tumor cells in the intermediate zone, were positive for TRAIL expression. The mean antigenic load for TRAIL in the intermediate zone was 22 (95% CI: 11-34) (Figure 3). No correlation could be found between survival and the antigenic load for TRAIL within the tumor specimens.

TRAIL-R1 expression

No difference in staining intensity of TRAIL-R1 was observed between the perinecrotic and intermediate zone. Forty-seven of the 62 tumors showed TRAIL-R1 expression within the intermediate zone (Table 1). Table 1 shows the mean antigenic load (AGL) of TRAIL-R1 within the intermediate area in relation to the different staining categories.

Quantitative measurement of the mean percentage of TRAIL-R1 expression on tumor cells in the intermediate zone was 19 % (95% CI: 12-26) (Figure 3A).A positive correlation was found between survival and the percentage of positive tumor cells expressing TRAIL-R1 (Spearman r: 0,25; 95% CI: -0.006 to 0.48; p=0,049 (Table 2)). The antigenic load of TRAIL-R1 expression was 39 (95% CI: 26-53) (Figure 3B). The antigenic load of TRAIL-R1 (Spearman r: 0,28; 95% CI: 0.030 to 0.50; p=0.024) (Figure 4 A; Table 2) was positively correlated with survival.

Univariate and multivariate analysis (Table3 and Fig 5) were performed to evaluate if TRAIL-R1 and R2 expression were independent factors for survival. Radiotherapy was significantly associated with survival in univariate and multivariate analyses. Therefore we excluded the patients who did not received radiotherapy after surgery and also performed a separate univariate and multivariate subgroup analyses on those patients who had received radiotherapy (n=47)

Analysis in the total study group (n=62) showed that the dichotomized staining intensity for TRAIL-R1 (0/1 vs. 2/3) (p=0.04), AGL TRAIL-R1 (p=0.016) and % of TRAIL-R1 expression on tumor cells (p=0.03) were all significantly associated with survival in univariate analysis (Table3). In the multivariate analysis the TRAIL-R1 factors were not shown to be separable prognostic markers for survival (Table 3).

However, multivariate analysis in the subgroup (patients who had received radiotherapy (n=47)) showed significance for two of the three parameters (staining TRAIL-R1 p=0.12; AGL TRAIL-R1 p=0.023; % TRAIL-R1 p=0.036)

TRAIL-R2 expression

In general, the semi-quantitative assessment showed a more intense staining pattern for TRAIL-R2 than for TRAIL-R1, although statistical significance was not reached. TRAIL-R2 expression (Table 1) was present in 57 of the sixty-two tumor specimens. The mean quantitative expression of TRAIL-R2 on tumor cells within the intermediate zone of the tumor was 30% (95% CI: 25-36) (Figure 3A). The percentage of tumor cells expressing TRAIL-R2 was significantly higher than TRAIL-R1 (p=0.005) (Figure 3A). The difference in antigenic load between TRAIL-R1 and TRAIL-R2 was statistically significant (p=0.01) (Figure 3B). The antigenic load for TRAIL-R2 was 56 (95% CI: 42-70). A positive correlation was found between survival and the antigenic load of TRAIL-R2 (Spearman r: 0,29; 95% CI: 0.030 to 0.51; p=0.022) (Figure 4 B).

Survival curves of the staining intensity or the antigenic load (AGL) within the subgroup are expressed in Figure 6.

Analysis in the total study group (n=62) showed that the dichotomized staining intensity for TRAIL-R2 (0/1 vs. 2/3) (p=0.009) and AGL TRAIL-R2 (p=0.003) were significantly

associated with survival in univariate analysis (Table3). The % of TRAIL-R2 expression on tumor cells (p=0.07) did not reach significance. In multivariate analysis, both dichotomized staining intensity and AGL TRAIL-R2 maintained significance (p=0.004 resp. p=0.03) and the % TRAIL-R2 expression was almost significant (p=0.059)(Table3).

Multivariate analysis within the subgroup showed that the parameters dichotomized staining TRAIL-R2 (p=0.002) and AGL TRAIL-R2 (p=0.004) were independent factors associated with survival. The % TRAIL-R2 expression (p=0.08) was not associated with survival.

RT-PCR analysis of TRAIL receptors in astrocytic tumors and normal brain

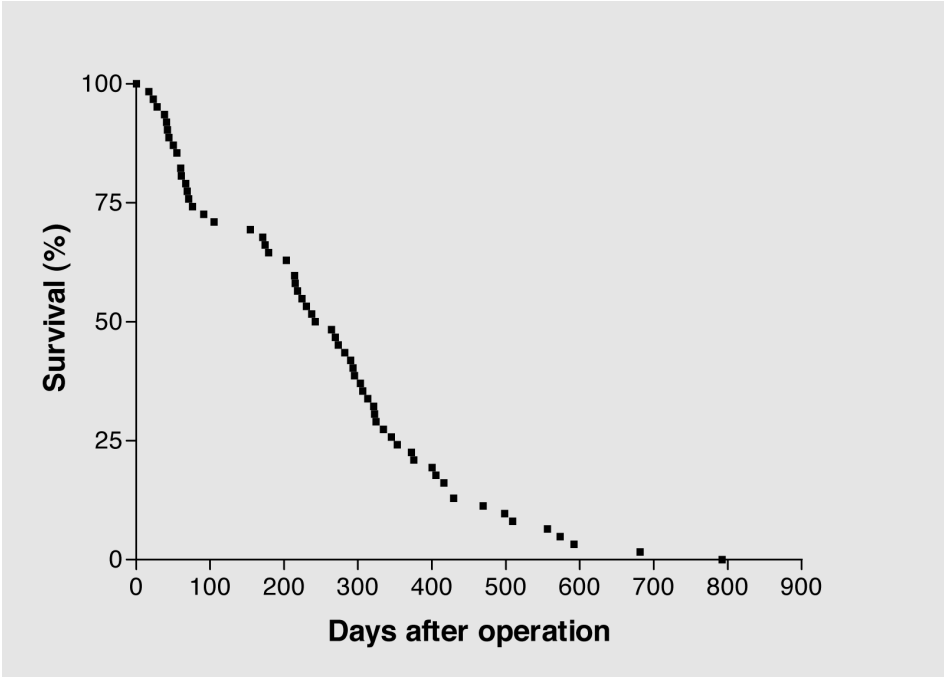
TRAIL-R1

TRAIL-R1 mRNA could be detected in all tissue samples tested for mRNA expression (Figure 7). RT-PCR showed a significant difference in mRNA expression of TRAIL-R1 within the five groups of primary brain tumors (Kruskal-Wallis-test; p=0.03) (Figure 8A). The primary GBM group showed a relative downregulation of TRAIL-R1 mRNA in compared to the other primary tumors and normal brain tissue. There seems to be an inverse relation between the mRNA expression and the WHO grade.

TRAIL-R2

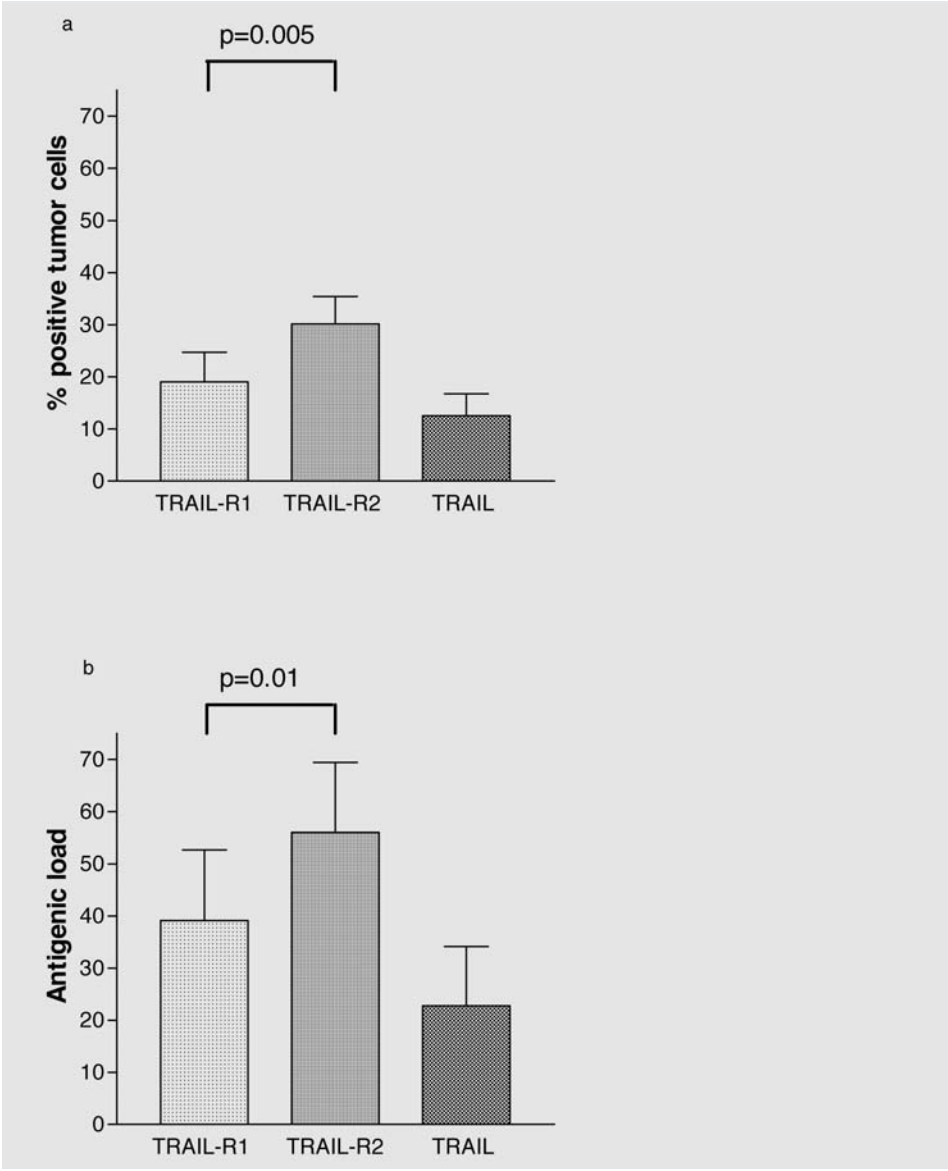
The splice variants of TRAIL-R2 were detected in all tumors (Figure 7), upregulation of either of the variants was not detected. The normalized level of TRAIL-R2 was a fraction higher in compared to TRAIL-R1 (Figure 8B). No significant difference in TRAIL-R2 mRNA could be detected among the various astrocytic tumors (p=0.07).

Figure 1



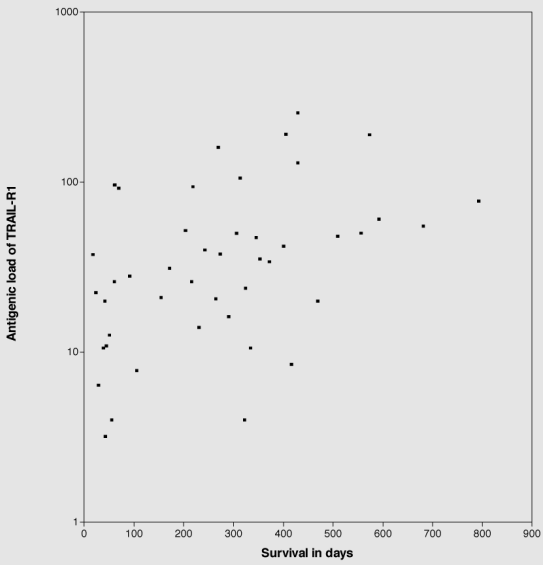
Kaplan Meier curve of overall survival of 62 patients with a glioblastoma multiforme. Mean survival is at 259 days.

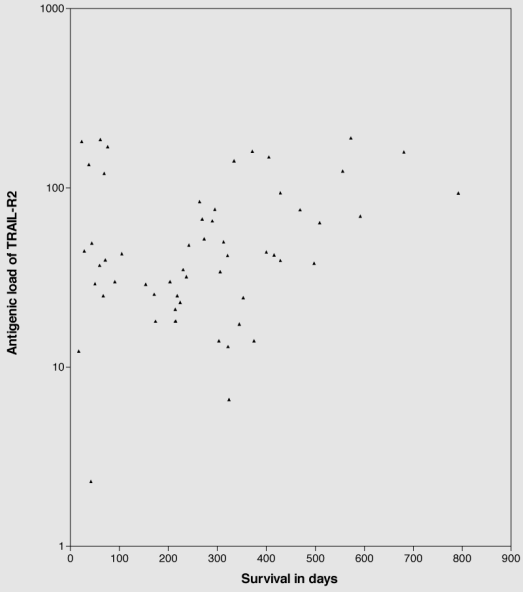
Figure 3



The percentage of positive tumor cells (a) of TRAIL-R1, TRAIL-R2 and TRAIL within the intermediate zone of the tumor tissue of patients with a GBM. The "antigenic load" (b) is the product of the staining intensity and the percentage of TRAIL-receptor or TRAIL positive tumor cells. The antigenic load is scaled from 0 to 300. For both parameters a significant difference between TRAIL-R1 and TRAIL-R2 expression could be found. Bars represent 95% CI.

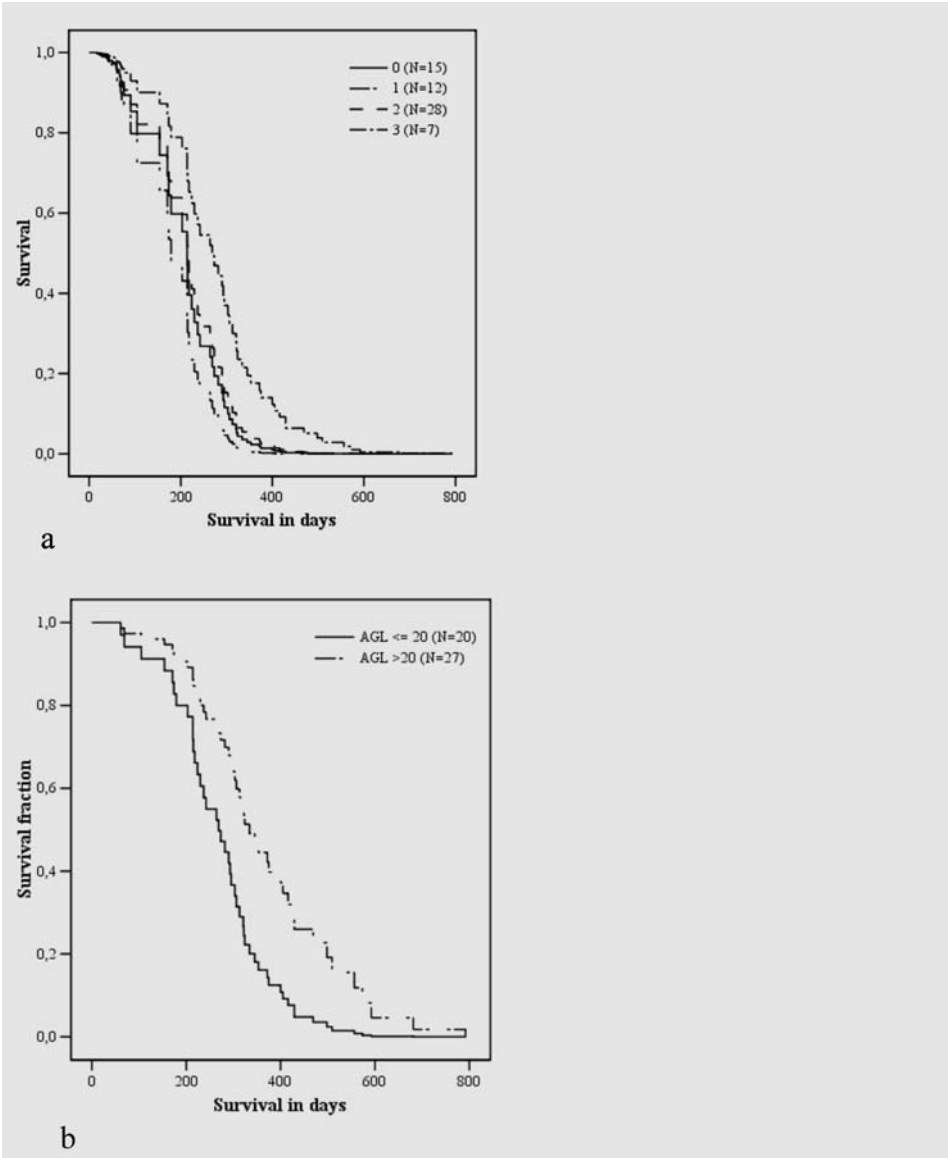
Figure 4





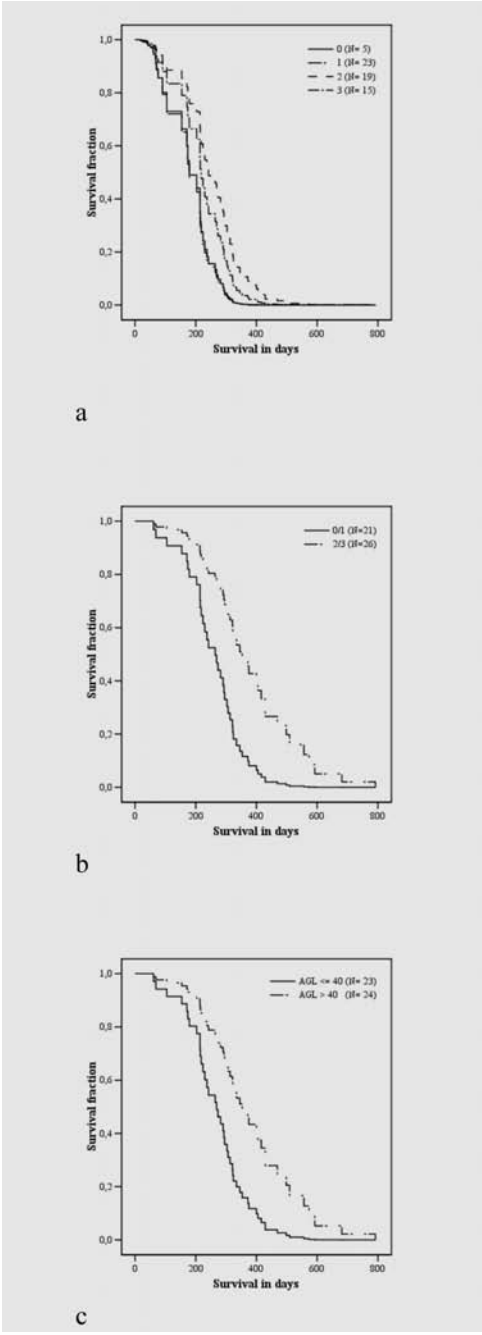
Correlation between survival and the antigenic load of TRAIL-R1 (Spearman r: 0,28; 95% CI: 0.030 to 0.50; p=0.024) and TRAIL-R2 (Spearman r: 0,29; 95% CI: 0.030 to 0.51; p=0.022).

Figure 5



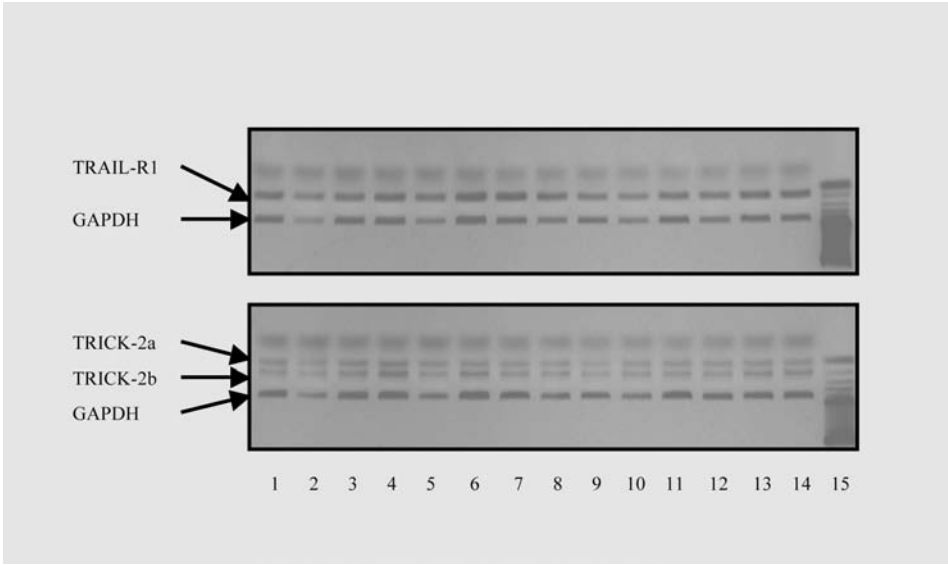
Cox proportional hazard survival curves bases on multivariate analysis of patients with a GBM with regard to TRAIL-R1 expression. (a) survival curves related to staining intensity for the total group. (b) dichotomized analysis of AGL ≤ 20 vs. > 20 within the subgroup (N=47) (p=0.023).

Figure 6



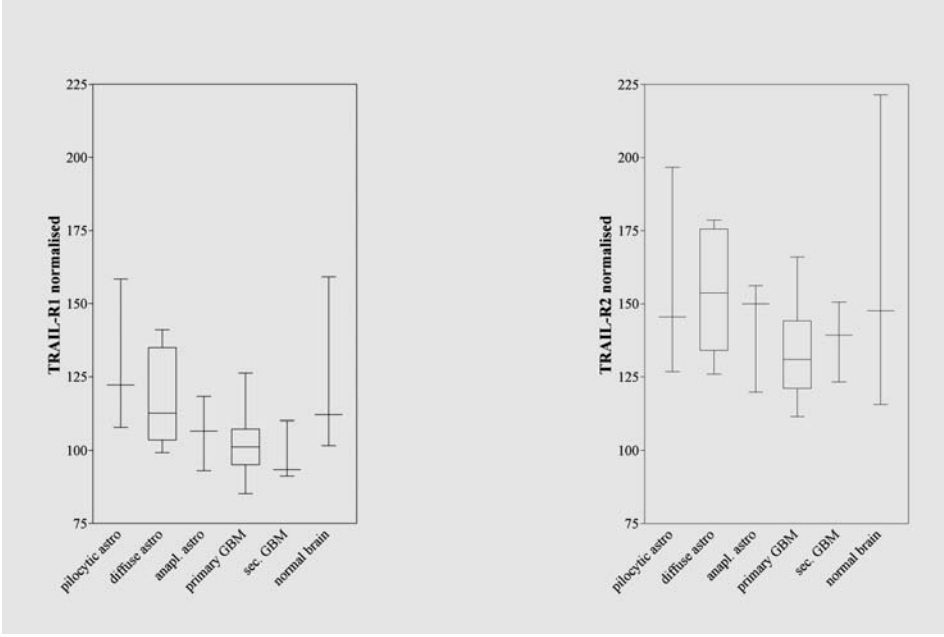
Cox proportional survival curves based on multivariate analysis of patients with a primary GBM with regard to TRAIL-R2 expression. (a) survival curves related to staining intensity for the total group (N=62). (b) survival curves (p=0.002) of low (0/1) versus high (2/3) staining intensity of TRAIL-R2 within the subgroup (N=47). (c) dichotomized analysis of AGL ≤ 40 versus > 40 within the subgroup (p=0.004).

Figure 7



Representative samples of mRNA expression of TRAIL-R1 (154 bp) and TRAIL-R2 splice variants (TRICK2a (93 bp) and TRICK-2b (181 bp)) of primary astrocytic tumors and normal brain tissue. Al samples showed TRAIL-R1 and TRAIL-R2 variant expression. The negative control (not shown) showed no expression. GAPDH (500 bp) was used as reference. pilocytic astrocytoma: lane 8; diffuse astrocytoma: lane 5, 10,12; anaplastic astrocytoma: lane 3 and 7; primary GBM: lane 4, 6, 13; secondary GBM: lane 11 and 14; normal brain tissue: lane 1, 2 and 9. Lane 15 is a 100 bp ladder.

Figure 8



RT-PCR expression of TRAIL-R1 and TRAIL-R2 in various astrocytic brain tumors and normal brain. A Kruskal-Wallis test between the various astrocytic groups showed a statistical significant difference for TRAIL-R1 (p=0.03). TRAIL-R2 mRNA is higher expressed in comparison to TRAIL-R1. No significant difference was found between the various grades of astrocytic tumors for TRAIL-R2 (p=0.07). GBM n=19; pilocytic astrocytoma n=3; diffuse astrocytoma n=6; anaplastic astrocytoma n=3; secondary GBM n=3 and normal brain tissue n=4. GAPDH was used as reference.

Table 1

Table 1: Expression pattern of TRAIL receptors in glioblastoma multiforme tumors within the intermediate zone

Staining category	TRAIL-R1 (n=62)	TRAIL-R1 (n=62) mean antigenic load	TRAIL-R2 (n=62)	TRAIL-R2 (n=62) mean antigenic load
0	15 (24, 2%)	0	5 (8,1%)	0
1	12 (19,4%)	12,9	23 (37,1%)	26,3
2	28 (45,1%)	54,5	19 (30,6%)	68,5
3	7 (11,3%)	106,5	15 (24,2%)	104,3

Table 2

Table 2: Correlation between survival and TRAIL receptor expression within the intermediate zone

	TRAIL-R1 (% pos. cells in tumor)	TRAIL-R2 (% pos. cells in tumor)	TRAIL-R1 (antigenic load)	TRAIL-R2 (antigenic load)
Survival	yes (p=0.049)	no(p=0.17)	yes(p=0.024)	Yes(p=0.022)

Table 3

Table 3 Univariate and multivariate analyses of survival in patients with a GBM

Total study group (n=62)	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	p	HR	95% CI	p
Age	1.02	0.99-1.04	0.10	1.02	0.99-1.04	0.15
Gender	1.11	0.64-2.00	0.71	1.15	0.64-2.04	0.64
Radiotherapy	75	15 - 328	0.000	72	15 - 357	0.000
Staining TRAIL-R1 0/1 vs. 2/3	1.75	1.04-3.03	0.04	1.47	0.84-2.58	0.18
Staining TRAIL-R2 0/1 vs. 2/3	2.10	1.20-3.66	0.009	2.36	1.31-4.23	0.004
AGL TRAIL-R1 ≤ 20 vs. >20	1.93	1.13-3.03	0.016	1.77	0.98-3.19	0.056
AGL TRAIL-R2 ≤ 40 vs. >40	2.30	1.30-4.00	0.003	1.90	1.05-3.40	0.03
% TRAIL-R1 expression	1.03	1.00-1.02	0.03	1.01	1.00-1.03	0.058
% TRAIL-R2 expression	1.01	1.00-1.03	0.07	1.02	1.00-1.03	0.059
Subgroup (n=47)						
Staining TRAIL-R1 0/1 vs. 2/3	1.64	0.88-3.05	0.12	1.67	0.88-3.20	0.12
Staining TRAIL-R2 0/1 vs. 2/3	2.77	1.40-5.35	0.002	2.95	1.50-5.80	0.002
AGL TRAIL-R1 ≤ 20 vs. >20	1.75	0.98-3.45	0.06	2.25	1.12-4.54	0.023
AGL TRAIL-R2 ≤ 40 vs. >40	2.50	1.33-4.79	0.004	2.56	1.34-4.89	0.004
% TRAIL-R1 expression	1	1.00-1.02	0.054	1.01	1.01-1.03	0.036
% TRAIL-R2 expression	1.01	1.00-1.03	0.056	1.01	1.00-1.03	0.08

Discussion

Multivariate analysis in the total study group did not associate TRAIL-R1 (staining intensity, AGL and % TRAIL-R1 expression) as an independent variable for survival.

Fifteen patients (24 %) of the total study group, rapidly deteriorated after neurosurgical debulking and for this reason these patients did not receive radiotherapy. Most of these patients had a KPS between 70 and 80 and in our hospital patients with a KPS between 70 and 80 are eligible for operation. The consequence of operating this patient group was the fact that a minor discomfort during hospital stay or after discharge lead to deterioration of the patient with dropping of the KPS under 70 and subsequently these patients were not eligible for further postoperative radiotherapy. The rapid deterioration preceded the start of the radiotherapy and with this in mind, not having had radiotherapy was not the reason for their rapid deterioration but other influences were. To eliminate the radiotherapy bias on the patient group, these patients were excluded in a multivariate subgroup analysis.

In the subgroup analysis TRAIL-R1 (AGL and % TRAIL-R1 expression) were independent variables for survival. The staining intensity was also in the subgroup analysis not associated with survival. Therefore the staining intensity alone cannot be used to categorize patients in a certain prognostic survival group. However measurement of the staining intensity can be of importance as an adjuvant parameter in relation to quantitative measurements such as the percentage positive tumor cells for TRAIL-R1 expression. The study results show that the antigenic load, used as a variable, in evaluation of TRAIL receptor expression, is more significant associated with survival than the individual semi-quantitative or quantitative parameters.

RT-PCR analysis detected an inverse correlation between the amount of TRAIL-R1 mRNA and the WHO grade of astrocytic tumors. Low grade astrocytic tumors showed a higher expression of TRAIL-R1 mRNA than high grade astrocytic tumors. The results of both the multivariate analysis and the RT-PCR indicate that TRAIL-R1 expression is a prognostic factor for survival in patients with a primary GBM. Sträter et al. found similar results in patients with a colon carcinoma [17].

Multivariate analysis of TRAIL-R2 (staining intensity and AGL) shows that these variables are independently associated with survival both in the total study group and in the subgroup.

As shown in Table 3, age was not independently associated with survival. Within this study only primary GBM's were subject of investigation. Secondary GBM were not included. Secondary GBM's tend to have a peak incidence at a younger age than primary GBM. Therefore our study population is older and because of the fact that they are all

primary GBM's, the standard deviation of the parameter age within the study group is small with the consequence that age is not associated with survival.

TRAIL-R1 is a death inducing receptor which is present on normal human tissue [18] and also on various tumors including brain neoplasm's [12]. Soluble TRAIL can bind to the TRAIL-R1 receptor, thereby inducing an intracytoplasmic cascade of effector proteins, which eventually leads to apoptosis [8,19]. Interestingly, apoptosis can be induced even at low concentrations of sTRAIL binding to TRAIL-R1. In that respect TRAIL-R1 could be of importance as a target for human anticancer TRAIL therapy.

Frank et al. [12] described TRAIL receptor expression in the cytoplasm of various tumors, including 5 glioblastoma specimens as well as in normal brain. They found TRAIL receptor expression on the mRNA level within every tumor specimen they investigated. We also detected TRAIL-R1 receptor expression on the mRNA level in 100% of the glioblastomas, other glial tumors and normal cerebral tissue. TRAIL-R1 mRNA was higher expressed in the low grade astrocytomas when compared to high grade tumors.

Through downregulation of TRAIL-R1 on both the protein and receptor level high grade glioma tumor cells might escape apoptosis induction from TRAIL-TRAIL receptor interaction.

In order to be informed about the actual receptor status of GBM tumor tissue, TRAIL receptor expression on the cell membrane should be assessed on the protein level. The IHC data in this study showed both cytoplasmic and, a more intense, membranous staining. The staining intensity in the intermediate zone was stronger than in the perinecrotic zone, although this difference was not significant. On the basis of the semi-quantitative IHC analysis, TRAIL-R1 was present in more than 75% of the GBM tumors. Morphometrical analysis showed a mean TRAIL-R1 expression in 19% of the tumor cells (95% CI: 13-25%) which was significantly correlated with survival. The antigenic load was also significantly positively correlated with survival.

TRAIL-R2 is like TRAIL-R1 a death inducing receptor. However, apoptosis induction through this receptor differs from that of TRAIL-R1. TRAIL-R2 can only be activated by very high levels of monomeric soluble TRAIL or crosslinked soluble TRAIL [20], while TRAIL-R1 can be activated by monomeric sTRAIL. These distinct apoptosis activating mechanisms between TRAIL-R1 and TRAIL-R2 may have consequences for treatment of GBM patients with soluble human TRAIL.

In general, semi-quantitative expression of TRAIL-R2 was more intense than TRAIL-R1, although statistical significance was not reached. TRAIL-R2 was expressed in more than 90% of the tumors. Furthermore, a positive correlation was found between tumor cell expression of TRAIL-R1 and TRAIL-R2. The quantitative and semi-quantitative analysis

of TRAIL-R2 did not correlate with survival. Surprisingly the antigenic load for TRAIL-R2, like TRAIL-R1, was significantly correlated with survival and seems of more importance as a prognostic parameter than the separate quantitative and semi-quantitative results.

A mean of 27% of the tumor cells was positive for TRAIL-R2 expression. The difference in percentage of tumor cells expressing TRAIL-R2 compared to TRAIL-R1 was statistically significant. The data presented in this study confirm the results of *in vitro* studies regarding the higher expression of TRAIL-R2 in GBM cell lines, compared with TRAIL-R1 [8]. The difference however is not as distinct as it is in the *in vitro* data. The percentage of TRAIL receptor expression in tumor tissue in this study is much lower compared with the *in vitro* data from tumor cell lines. In GBM cell lines, 58 to 99% TRAIL-R2 positive cells were observed using FACS analysis [8]. No relevant TRAIL-R1 expression in these GBM cell lines was found. One could argue the relevancy of the amount of TRAIL receptor expression in cell lines that are homogenous tumor cells and therefore not representative for the heterogeneous phenotype of primary GBM tissue. Another argument explaining the difference, in expression level of both receptors, between the in vitro data and our data may be due to the method of detection. Flow cytometric evaluation is more sensitive to detect TRAIL receptor expression on tumor cell membranes than immunohistochemistry. In that respect FACS analysis of cell suspensions of primary tumor tissue could give much more insight in the death inducing receptor expression on primary GBM tumor tissue.

Like Frank et al. [12] we also found in all of the investigated tissue specimens (glioma tumor tissue and normal brain tissue) TRAIL-R2 mRNA expression and the TRAIL-R2 transcript variants TRICK-2a and TRICK-2b. In contrast with TRAIL-R1 mRNA expression, no correlation of TRAIL-R2 mRNA expression, with survival could be found.

The relatively low percentage of TRAIL-R1 and TRAIL-R2 positive tumor cells raises the question, whether treatment of glioblastomas with TRAIL alone will be sufficient, or that combination therapies might be more effective. From the study of Stupp et al. [2] we can conclude that, Temozolomide as an adjuvant to radiotherapy, prolongs overall survival with 2 to 3 months. As with Temozolomide we might suspect that TRAIL therapy also prolongs survival particularly in glioblastomas with a high percentage of TRAIL receptors and therefore TRAIL can be of importance as an adjuvant therapy besides radiotherapy and chemotherapy.

Finally we analyzed TRAIL expression within GBM tumor tissue. TRAIL is also endogenously expressed within various tumors. The presence of TRAIL in tumors and its absence in normal brain tissue is not fully understood [21, 22]. Rieger et al. [23] stated that TRAIL expression on tumor cells may be a part of the defense system of glioma cells to mediate tumor immune escape, as has been suggested for the CD95 ligand. Membranous TRAIL expression could be used as a protective shield against activated

human T cells that want to attack the immunogenic tumor cell. TRAIL expressed on tumor cells could bind to TRAIL receptors on T cells and subsequently induce apoptosis in these T cells and thereby inactivating the immune defense system. In this study TRAIL expression was shown in 85% of the tumors and a mean of 13% TRAIL positive tumor cells was found. There was a stronger staining intensity of TRAIL within the perinecrotic zone in comparison with the intermediate zone, and this difference was found to be statistically significant (p=0.0001). This result differs from data showing that FasL, like TRAIL a member of the TNF family, was present on glioma cells but without preferential perinecrotic distribution [24].

In two publications [21,23] semi-quantitative TRAIL expression on human brain tumors using immunohistochemistry is described. In the astrocytoma group grade 1 to 4, 100% of these tumors were positive for TRAIL.

Soluble TRAIL is an apoptosis inducing ligand and one of the TNF related ligands. Other ligands belonging to this family are TNF-alpha and FasL. All three ligands can induce apoptosis. However therapeutic systemic levels of FasL and TNF-alpha were found to be lethally toxic in humans and in animals [25,26]. TRAIL was studied in rodents and in non-human primates and showed no toxicity [3]. Because of its favorable toxicity profile and its apoptosis inducing potential in various glioblastoma cell lines it seems to be a suitable antineoplastic agent.

However, the widespread expression of TRAIL receptors throughout the human body and the uncertainty about TRAIL-related toxicity towards certain normal cells and tissues [5,6] and also the relative low expression of TRAIL-receptors on glioblastoma tissue might hamper its clinical development. To prevent toxicity towards normal tissue it is of great importance to deliver TRAIL as selectively as possible to tumor tissue with a maximal mean tolerable dose (MTD). It could well be that the MTD for TRAIL is high enough to induce an apoptotic signal through the TRAIL-R1 receptor but too low for activation of the TRAIL-R2 receptor. A possible solution to bypass systemic toxicity of TRAIL and to optimize its apoptosis inducing potential for both receptors is targeted cell restricted enhanced apoptosis induction through a scFv:sTRAIL protein with specificity for a preselected tumor cell surface target antigen. Recently it was demonstrated that augmentation of the therapeutic value of TRAIL can be achieved by increasing its tumor selective binding properties through genetic fusion to a tumor-selective antibody fragment (scFv) [20,27]. Primary glioblastomas selectively overexpress the EGFR and the concept of targeting TRAIL to this receptor could be applicable in glioblastoma treatment [28]. Through this concept TRAIL can be targeted in a specific way to the tumor and thereby bypassing normal tissues expressing also TRAIL receptors. In this way systemic toxicity can be minimized. Another advantage of the target cell restricted apoptosis induction concept is the fact that both TRAIL-R1 and TRAIL-R2 are activated at the same time although both receptors have a distinct apoptosis induction mechanism. This

study shows that TRAIL-R2 is more expressed within GBM tumor tissue than TRAIL-R1. Therefore TRAIL-R2 is an important target for TRAIL therapy. Because of its dependence on crosslinked TRAIL in order to induce apoptosis, the target cell restricted apoptosis induction through a scFv:sTRAIL protein might be of great importance in future TRAIL therapy.

Since we do not know how many tumor cells must be positive for TRAIL receptors to induce enough cell kill for tumor reduction, future clinical trials with TRAIL or scFv:sTRAIL proteins in GBM patients should include assessment of the TRAIL receptor status and cell surface target antigen levels on tumor tissue in order to optimize the therapeutic use of TRAIL in the individual patient.

References

1. DeAngelis, L. M.: Brain tumors. N Engl J Med 344: 114-123, 2001
2. Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., and Mirimanoff, R. O.: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005
3. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H.: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104: 155-162, 1999
4. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and .: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673-682, 1995
5. Nitsch, R., Bechmann, I., Deisz, R. A., Haas, D., Lehmann, T. N., Wendling, U., and Zipp, F.: Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). Lancet 356: 827-828, 2000
6. Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Billiar, T. R., and Strom, S. C.: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med 6: 564-567, 2000
7. de Vries, E. G., van der Graaf, W. T., Heijenbrok, F. J., Hoekstra, H. J., and de Jong, S.: Don't blaze the trailblazer TRAIL too early. Lancet 356: 2014-2000
8. Knight, M. J., Riffkin, C. D., Muscat, A. M., Ashley, D. M., and Hawkins, C. J.: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells.
9. Grotzer, M. A., Eggert, A., Zuzak, T. J., Janss, A. J., Marwaha, S., Wiewrodt, B. R., Ikegaki, N., Brodeur, G. M., and Phillips, P. C.: Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. Oncogene 19: 4604-4610, 2000
10. Dorr, J., Waiczies, S., Wendling, U., Seeger, B., and Zipp, F.: Induction of TRAIL-mediated glioma cell death by human T cells. J Neuroimmunol 122: 117-124, 2002
11. Rohn, T. A., Wagenknecht, B., Roth, W., Naumann, U., Gulbins, E., Krammer, P. H., Walczak, H., and Weller, M.: CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. Oncogene 20: 4128-4137, 2001
12. Frank, S., Kohler, U., Schackert, G., and Schackert, H. K.: Expression of TRAIL and its receptors in human brain tumors. Biochem Biophys Res Commun 257: 454-459, 1999
13. Kleihues P and Cavenee WK: Pathology & Genetics of Tumours of the Nervous System. 2000
14. Sondergaard, K. L., Hilton, D. A., Penney, M., Ollerenshaw, M., and Demaine, A. G.: Expression of hypoxia-inducible factor 1alpha in tumours of patients with glioblastoma. Neuropathol Appl Neurobiol 28: 210-217, 2002
15. Plate, K. H., Breier, G., Weich, H. A., and Risau, W.: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359: 845-848, 1992
16. Shweiki, D., Itin, A., Soffer, D., and Keshet, E.: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359: 843-845, 1992
17. Strater, J., Hinz, U., Walczak, H., Mechtersheimer, G., Koretz, K., Herfarth, C., Moller, P., and Lehnert, T.: Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. Clin Cancer Res 8: 3734-3740, 2002
18. Spierings, D. C., de Vries, E. G., Vellenga, E., van den Heuvel, F. A., Koornstra, J. J., Wesseling, J., Hollema, H., and de Jong, S.: Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem 52: 821-831, 2004

19. Golstein, P.: Cell death: TRAIL and its receptors. Curr Biol 7: R750-R753, 1997
20. Wajant, H., Moosmayer, D., Wuest, T., Bartke, T., Gerlach, E., Schonherr, U., Peters, N., Scheurich, P., and Pfizenmaier, K.: Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 20: 4101-4106, 2001
21. Nakamura, M., Rieger, J., Weller, M., Kim, J., Kleihues, P., and Ohgaki, H.: APO2L/TRAIL expression in human brain tumors. Acta Neuropathol (Berl) 99: 1-6, 2000
22. Dorr, J., Bechmann, I., Waiczies, S., Aktas, O., Walczak, H., Krammer, P. H., Nitsch, R., and Zipp, F.: Lack of tumor necrosis factor-related apoptosis-inducing ligand but presence of its receptors in the human brain. J Neurosci 22: RC209-2002
23. Rieger, J., Ohgaki, H., Kleihues, P., and Weller, M.: Human astrocytic brain tumors express AP02L/TRAIL. Acta Neuropathol (Berl) 97: 1-4, 1999
24. Gratas, C., Tohma, Y., Van Meir, E. G., Klein, M., Tenan, M., Ishii, N., Tachibana, O., Kleihues, P., and Ohgaki, H.: Fas ligand expression in glioblastoma cell lines and primary astrocytic brain tumors. Brain Pathol 7: 863-869, 1997
25. Tartaglia, L. A. and Goeddel, D. V.: Two TNF receptors. Immunol Today 13: 151-153, 1992
26. Nagata, S.: Apoptosis by death factor. Cell 88: 355-365, 1997
27. Bremer, E., Kuijlen, J., Samplonius, D., Walczak, H., de Leij, L., and Helfrich, W.: Target cell-restricted and -enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. Int J Cancer 109: 281-290, 2004
28. Bremer, E., Samplonius, D. F., van Genne, L., Dijkstra, M. H., Kroesen, B. J., de Leij, L. F., and Helfrich, W.: Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. J Biol Chem 280: 10025-10033, 2005

Chapter 4

Target cell restricted and enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma associated antigen EGP2

Edwin Bremer[1], Jos Kuijlen[2], Douwe Samplonius[1], Henning Walczak[3], Lou de Leij[1] and Wijnand Helfrich[1]

[1]Groningen University Institute for Drug Exploration (GUIDE), Department of Pathology & Laboratory Medicine, Laboratory for Tumor Immunology
[2]Department of Neurosurgery, University Medical Center Groningen, The Netherlands
[3]Division of Apoptosis Regulation, Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany

Int J Cancer. 2004;109(2):281-90

Abstract

The apparent tumor selective apoptosis inducing activity of recombinant soluble TRAIL has aroused much interest for use in clinical application. However, to fully exploit its therapeutic potential the characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. Firstly, the wide spread expression of the various TRAIL receptors throughout the human body; secondly, the differential binding affinities and cross-linking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2; and thirdly, the solution behavior of particular sTRAIL preparations. Therefore, we constructed a novel TRAIL fusion protein, designated scFvC54:sTRAIL, comprising the human scFv antibody fragment C54 genetically linked to the N-terminus of human soluble TRAIL. The scFvC54:sTRAIL fusion protein was designed to induce apoptosis by cross-linking of agonistic TRAIL receptors only after specific binding of scFvC54:sTRAIL to the abundantly expressed carcinoma-associated cell surface antigen EGP2 (alias EpCAM). Target antigen restricted apoptosis induction was demonstrated for various EGP2-positive tumor cells and could be inhibited by an EGP2 competing antibody. Target antigen binding converted soluble scFvC54:sTRAIL into a membrane bound form of TRAIL that was capable of signaling apoptosis not only through TRAIL-R1, but also through TRAIL-R2. Size-exclusion FPLC indicated that scFvC54:sTRAIL was produced as stable and homogeneous trimers in the absence of detectable TRAIL aggregates. The favorable characteristics of the scFvC54:sTRAIL fusion protein potentially reduce the amount of sTRAIL required for anti-tumor activity and may be of value for the treatment of various human carcinomas.

Introduction

The specific susceptibility of tumor cells to the pro-apoptotic activity of TRAIL, and the apparent lack of susceptibility of normal cells, make this molecule a promising anti-cancer therapeutic agent. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL), but can also be proteolytically cleaved to form a soluble trimer (sTRAIL)[1,2]. To date, various forms of soluble recombinant TRAIL have been generated, including FLAG-, HIS-, and non-tagged sTRAIL variants, all of which induce apoptosis in a wide range of human tumor cell lines[3]. Potent anti-tumor activity of various sTRAIL variants has been demonstrated in several mouse xenograft models of human cancers, including colorectal cancer[3,4], glioblastoma[4], and breast cancer[5].

Both memTRAIL and sTRAIL can interact with the agonistic TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 that initiate apoptosis via their intracellular death domains[6,7,8,9]. TRAIL also binds, albeit with lower affinity[10], to TRAIL-R3/DcR1and TRAIL-R4/DcR2[7,11,12,13], both of which lack a functional death domain. TRAIL-R3 and TRAIL-R4 are considered to act as receptors that potentially modulate TRAIL activity.

Expression of the different TRAIL receptors has been demonstrated not only on various tumors, but also on a wide variety of normal human tissues, indicating that apoptosis induction by TRAIL is a delicately regulated mechanism much of which is still elusive. Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the Death Inducing Signaling Complex (DISC)[14,15,16,17] by recruitment of the adapter protein FADD and resultant binding and activation of initiator caspases-8 and -10[18,16,19,20,21,19]. Activated caspase-8 and -10 subsequently activate downstream effector caspases, including caspase-3, -6 and -7, which cleave cytoskeletal and nuclear proteins essential for cell survival such as PARP, alpha-Fodrin, DFF and Lamin A, resulting in apoptosis.

Formation of the TRAIL receptor DISC is strongly enhanced when aggregated or complexed TRAIL binds to TRAIL-R1 or TRAIL-R2. Furthermore, TRAIL-R1 and -R2 were shown to have rather distinct cross-linking requirements for the initiation of apoptosis[22]. Both sTRAIL and memTRAIL can efficiently activate TRAIL-R1 even at low concentrations, whereas TRAIL-R2 can only be activated by memTRAIL or recombinant sTRAIL that is secondarily cross-linked by antibodies. Previously, Wajant et al.[23] demonstrated that signaling capacity of sTRAIL for TRAIL-R2 could be restored by genetic fusion to a recombinant antibody fragment (scFv) recognizing the tumor stroma marker fibroblast activation protein (FAP).

Independently, we developed a versatile expression system in CHO cells for the rapid construction and evaluation of scFv:sTRAIL fusion protein variants. Here we present a detailed characterization of a novel scFv:sTRAIL fusion protein that selectively targets the pancarcinoma associated membrane antigen EGP2 (also known as GA733-2,

EpCAM or CO17-1A antigen). EGP2 is a well-established target antigen that is overexpressed on the cell surface of various human carcinomas such as colorectal, breast, and small cell lung carcinoma[24,25]. In normal epithelia EGP2 expression is limited to the baso-lateral membrane [26,27]. Furthermore, EGP2 is not shed into the circulation and has been extensively studied in antibody-mediated imaging and immunotherapy of human carcinomas[28,29,30]. This is the first report on the construction and detailed characterization of a scFv:sTRAIL fusion protein with specificity for the therapeutically relevant pancarcinoma associated target antigen EGP2/EpCAM.

Materials & Methods

Monoclonal antibodies and scFv antibody fragment

MAb MOC31 is a murine IgG1 with high affinity for human EGP2[31]. The anti-EGP2 scFvC54 has been previously selected from a large semisynthetic phage display library with random human VH -VL pairings and has a VH-(G4S)3-VLk format[32]. MAb MOC31 and scFvC54 compete for binding to the same epitope on the extracellular domain of EGP2. TRAIL activity neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands). MAb 2E5 neutralizes TRAIL activity by binding to an epitope on the extracellular domain of TRAIL that inhibits binding to the various TRAIL receptors. A multimeric form of the extracellular domain of EGP2 (sEGP2) was produced and purified as described previously[33]. Multimeric sEGP2 was used to secondarily cross-link scFvC54:TRAIL.

Cell lines

The following cell lines were purchased from the ATCC: A172 and U87MG (both astrocytoma grade IV), SW948 (colorectal adenocarcinoma), Chinese Hamster Ovary (CHO-K1) and Jurkat (human ALL T-cell line). Jurkat cells express high levels of TRAIL-R2 on the cell surface but do not express detectable amounts of TRAIL-R1. As a result, Jurkat cells are only sensitive to cross-linked or aggregated sTRAIL preparations[22,34]. All cell lines were cultured in their respective media supplemented with 10% FCS at 37ºC in humidified 5% CO2 atmosphere, unless indicated otherwise.

EGP2 transduced cell lines

EGP2 transduced cell lines A172.EGP2, U87MG.EGP2, and Jurkat.EGP2 were generated by infection of the respective parental cell lines with retroviral particles encoding both EGP2 and Enhanced Green Fluorescent Protein (EGFP). In short, EGP2 cDNA was cloned into a retroviral vector derivative of LZRS-pBMN-lacZ[35] kindly provided by Dr. G. Nolan (Stanford University School of Medicine, San Francisco, CA), yielding LZRS-EGP2-IRES-EGFP. To produce retroviral particles, LZRS-EGP2-IRES-EGFP was transfected into the amphotrophic packaging cell line Phoenix, using Fugene-6 transfection reagent according to manufacturer's recommendations (Roche Diagnostics, Almere, The Netherlands). Transfected cells were selected by culturing in the presence of 1 μg/ml puromycin, 300 μg/ml hygromycin, 1 μg/ml diphtheria toxin (BD Biosciences Clontech, Palo Alto, USA). Viral particle-containing supernatant was harvested after 3 days and stored at –80°C until further use. Jurkat, A172 and U87MG cells (0.5 x 10^6 cells) were transduced with 1 ml supernatant containing 1x10^6 retroviral particles.

Supernatants were removed after overnight incubation, after which cells were transferred to fresh medium. Transduced cells were selected, both for EGFP-fluorescence and EGP2 expression (MAb MOC31-PE), using the MoFlo high-speed cell sorter (Cytomation, Fort Collins, USA).

Analysis of membrane expression of TRAIL-receptors and EGP2

Differential expression of TRAIL-R1, -R2, -R3, and -R4 on the various cell lines was assessed by flow cytometry using TRAIL receptor specific MAbs (Alexis). In short, cells were harvested and washed with serum free medium and resuspended at a concentration of 5x105 cells in 100 μl fresh medium containing the respective anti-TRAIL-R MAbs. Specific binding was detected using secondary PE-conjugated antibodies (DakoCytomation, Glostrup, Denmark). EGP2 expression on the tumor cell surface was analyzed by incubation with MOC31-PE. All antibody incubations were carried out for 45 minutes at 0°C and were followed by two washes with serum free medium.

Construction of scFvC54:sTRAIL

Eukaryotic expression plasmid pEE14scFv:sTRAIL was generated for the rapid construction, evaluation, and stable expression of scFv:sTRAIL fusion proteins in CHO-K1 cells. Plasmid pEE14scFv:sTRAIL is based on a vector we described earlier[36]. Important features of this novel vector are the presence of the murine kappa light chain leader peptide encoded upstream of two multiple cloning sites (MCS) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in the production cell line CHO-K1[37]. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs the produced fusion protein through the ER and Golgi complex resulting in excretion of fusion protein into the culture supernatant. In the first MCS, a 730 bp DNA fragment encoding scFvC54 derived from phagemid pHENscFvC54, was directionally inserted using the unique *Sfi*I and *Not*I restriction enzyme sites. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes XhoI and HindIII. TRAIL cDNA truncation was performed by PCR with proofread DNA polymerase according to standard protocol using primers T1: 5'- ATCCTCGAGTCTAGTGGTAGCGGAACCTCTGAGGAAACCATTT-3' (XhoI site is underlined) and T2: 5'-CCCAAGCTTCAGGTCAGTTAGCCAACTAAAAAG-3 (*Hind*III site is underlined). Figure 1A depicts a schematic presentation of the monomeric form of the scFvC54:sTRAIL fusion protein.

Production of scFvC54:sTRAIL in CHO-K1 cells

CHO-K1 cells were transfected with plasmid pEE14scFvC54:sTRAIL using the Fugene 6 reagent (Roche). Stable transfectants were generated by the glutamine synthetase selection method, essentially as described previously[37]. Briefly, pEE14scFvC54:sTRAIL transfected CHO-K1 cells were cultured in GMEM medium (First Link Ltd, West Midlands, UK) supplemented with 5% dialyzed fetal calf serum (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) and 100 µM L-methionine-sulfoximine (MSX) (Sigma). Individual clones, obtained after single cell sorting using the Moflo high speed cell sorter, were analyzed for stable and high expression of scFvC54:sTRAIL in the absence of MSX using a solid phase sandwich TRAIL ELISA kit according to manufacturer's recommendations (Diaclone SAS, Besançon, France). The procedure identified a recombinant CHO-K1 production cell line clone designated 70C1, that stably secreted scFvC54:sTRAIL into the medium at a concentration of 3,44 µg/ml. Large scale production of scFvC54:sTRAIL fusion protein was performed by culturing cell line 70C1 in roller bottles (Greiner Bio-One GmbH, Frickenhausen, Germany) at 37ºC in serum free CHO-S SFM II suspension medium (Gibco, Life Technologies b.v. Breda, The Netherlands) to a density of 5.0x106 cells/ml, after which supernatant was harvested (1500xg, 10 min) and stored at -80°C until further use.

Immunoblot analysis of scFvC54:sTRAIL fusion protein

Supernatant derived from scFvC54:sTRAIL production cell line 70C1 was separated by non-reducing SDS/PAGE (12% acrylamide) without sample boiling and was subsequently electroblotted to nitrocellulose (NC). Detection of scFvC54:sTRAIL fusion protein was performed by incubation with anti-TRAIL MAb 2E5 and secondary HRPO-conjugated Goat anti Mouse antibody (DAKO), after which specific binding was visualized using chemoluminescence (Roche). Soluble Flag-tagged TRAIL (Alexis) was used as control at a final concentration of 1 µg/ml. All antibody incubations were performed at room temperature in PBS/5% skim milk for 1,5 h and were followed by 3 washes with PBS/0,1%TWEEN.

Size-exclusion FPLC of scFvC54:sTRAIL

The solution behavior of scFvC54:sTRAIL was analyzed by size exclusion (SE) FPLC using a calibrated HiLoad 16/60 Superdex 200 Prep grade column (Amersham Biosciences AB, Upsala, Sweden) with a bed volume of 120 ml. Five ml supernatant derived from cell line 70C1 was loaded onto the column after which individual samples were collected at 3-minute intervals. All samples were analyzed for their capacity to induce apoptosis using the TRAIL sensitive cell line SW948. Furthermore, all samples were subjected to a sensitive TRAIL specific ELISA to quantitate the scFvC54:sTRAIL content.

Target antigen restricted cell surface binding of scFvC54:sTRAIL

Target antigen restricted binding of scFvC54:sTRAIL to the cell surface was assessed by flow cytometry using cell lines SW948 (EGP2-positive) and Jurkat (EGP2-negative). In short, cells were harvested and washed with serum free medium. Subsequently, cells were incubated with scFvC54:sTRAIL (300 ng/ml) in the presence or absence of either the EGP2 competing antibody MOC31 (7,15 µg/ml) or the TRAIL activity neutralizing MAb 2E5 (1 µg/ml). Detection of cell surface bound scFvC54:sTRAIL was performed using anti-TRAIL-PE (Diaclone SAS). All antibody incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

Target antigen restricted apoptosis induction by scFvC54:sTRAIL

Target antigen restricted apoptosis induction by scFvC54:sTRAIL (300 ng/ml) was assessed by analysis of the following apoptosis related cellular phenomena: tumor cell viability, phosphatidyl serine exposure on the outer cell-membrane surface, caspase-8 and -3 activation, DFF degradation by activated caspase 3, and DNA fragmentation. The different analyses were performed in the presence or absence of either MOC31 (7,15 µg/ml) or 2E5 (1 µg/ml) and are described in more detail below.

Viability assay

Tumor cell viability was assessed by MTS assay (Promega Benelux b.v., Leiden, The Netherlands). Briefly, cells were seeded in flat bottom 96-well micro culture plates at a density of $3x10^4$ cells/well in 100 µl medium. After overnight culture, spent medium was removed and replaced by 200 µl medium containing the various experimental conditions. After 16 h, MTS assay was performed according to manufacturer's recommendations. Each experimental and control group consisted of six independent wells.

Immunoblot analysis of apoptosis

After treatment of SW948 cells with scFvC54:sTRAIL, intracellular apoptotic features were detected by incubation with antibodies against active caspase-8, active caspase-3 and DFF (PharMingen, San Diego, USA). Briefly, 2.5×10^6 tumor cells were seeded in 6 well plates and treated for 1, 2, 3, 5, 6, 12, and 24 h respectively with the various experimental conditions indicated in Figures 4A and B. Cells were collected by centrifugation (2,000xg; 10 min), lysed in lysis buffer (20 mM Tris-HCl, 5.0 mM EDTA, 2.0 mM EGTA, 100 mM NaCl, 0,05% SDS, 0,50% NP-40, 1 mM PMSF, 10 µg/ml Aprotein, 10 µg/ml Leupeptin), and subsequently sonicated on ice for 2x5sec. Subsequently, cleared super-

natants were collected by centrifugation (15.000xg, 10min) and separated by SDS-PAGE (12% AA) under reducing conditions and transferred to NC by electroblotting. Blots were incubated with the appropriate primary monoclonal antibodies and HRPO-conjugated secondary antibody. Specific binding was visualized by chemoluminescence (Roche). All antibody incubations were carried out for 1,5 h in PBS/5% skim milk and were followed by 3 washes with PBS/0,1%Tween.

Flowcytometric analysis of apoptosis induction by scFvC54:sTRAIL

Cells were harvested and resuspended in fresh medium at $1x10^6$ cells/ml and incubated for 16 h with the various experimental conditions. After 16 h, cells were harvested by centrifugation (1200 rpm, 5 min.) and analyzed with the AnnexinV-FITC/PI kit (IQ-products, Groningen, The Netherlands), the caspscreen flow cytometry apoptosis detection kit (Biovision, Mountain View, USA) and the single strand DNA specific MAb F7-26 (Alexis). AnnexinV-FITC/PI staining was performed according to manufacturer's recommendations and identifies early apoptotic cells with phosphatidyl serine exposure on the outer cell membrane and late apoptotic AnnexinV-FITC/PI double positive cells.

Caspase activation by scFvC54:sTRAIL was analyzed using the Caspscreen flow cytometry apoptosis detection kit that detects cleavage of the non-fluorescent substrate (aspartyl)2-Rhodamine 110 (D_2R) into fluorescent Rhodamine 110 by activated caspases.

DNA-fragmentation was analyzed with MAb F7-26 according to manufacturer's recommendations. MAb F7-26 specifically detects apoptotic DNA fragmentation and is based on the high sensitivity of DNA to thermal denaturation in condensed chromatin of apoptotic cells. MAb F7-26 specifically detects deoxycytidine in ssDNA of at least 25-30 bases in length in the absence of any reactivity to double-stranded DNA.

Results

Construction of scFvC54:sTRAIL

DNA encoding the extracellular domain of TRAIL (sTRAIL) was generated by PCR using proofread DNA polymerase. The resulting 593 bp PCR product encoded amino acids 95 - 281 of TRAIL, including cysteine residue number 230 (original position in memTRAIL), which is essential for the structure and activity of TRAIL[38]. The DNA fragment encoding scFvC54 and the sTRAIL PCR product were subsequently inserted in the first and second MCS of eukaryotic expression vector pEE14, yielding plasmid pEE14-scFvC54:sTRAIL. Sequence analysis confirmed the correct and in frame fusion of the scFvC54 and sTRAIL encoding DNAs (data not shown).

Eukaryotic production of scFvC54:sTRAIL

CHO-K1 cells transfected with pEE14-scFvC54:sTRAIL were selected for stable and amplified secretion of scFvC54:sTRAIL using the GS selection method and a TRAIL-specific ELISA. This procedure identified the producer CHO-K1 clone designated 70C1 that secreted 3,44 µg/ml fusion protein into the culture medium. Immunoblot analysis of a 70C1 culture medium sample identified scFvC54:sTRAIL as a fusion protein with an apparent molecular weight of approximately 52 kDa (Fig.2A, lane 1). This is in close proximity of the molecular weight of 51180 Da calculated for monomeric scFvC54:sTRAIL. Flag-tagged sTRAIL was detected as a 25 kDa monomer (Fig.2C, lane 2). The observed molecular weight of scFvC54:sTRAIL is the result of the genetic fusion of scFvC54 (26.6 kDa), 26 amino acid linker (2.6 kDa) and sTRAIL (22 kDa). In addition to monomeric scFvC54:sTRAIL two other bands were detected corresponding to dimeric (102 kDa) and trimeric (154 kDa) forms of scFvC54:sTRAIL. The latter bands were not observed when SDS/PAGE was performed under reducing conditions with sample boiling (data not shown).

Target antigen restricted binding of scFvC54:sTRAIL

Incubation of SW948 cells (EGP2 pos.) with scFvC54:sTRAIL resulted in strong and specific binding of scFvC54:sTRAIL to the cell surface (Fig.2B). The observed binding was target antigen specific since pre-incubation with the EGP2 blocking MAb MOC31 completely inhibited scFvC54:sTRAIL binding (Fig.2B). Cell surface binding of scFvC54:TRAIL via its TRAIL domain to tumor cells was minimal, as exemplified for Jurkat cells (EGP2 neg.) to which weak TRAIL domain mediated binding was observed (Fig.2C, solid line). This binding could be completely inhibited by co-incubation with MAb 2E5 (Fig. 2C, dashed line).

Target antigen restricted apoptosis induction by scFvC54:sTRAIL

Treatment with scFvC54:sTRAIL resulted in prominent apoptosis induction (as measured by a strong reduction of cell viability) in all the EGP2-positive tumor cell lines tested (Fig.3A), whereas viability was only minimally reduced in the EGP2-negative cell lines (Fig.3B). Induction of apoptosis by scFvC54:sTRAIL was target antigen dependent since pre-incubation with saturating amounts of MAb MOC31 restored cell viability to medium control in all EGP2-positive cell lines (Fig.3A). Co-incubation of SW948 cells with a fixed concentration of scFvC54:sTRAIL and increasing concentrations of MAb MOC31 resulted in a dose-dependent reduction in apoptosis (Fig.3C). Similar results were obtained for all other EGP2-positive tumor cell lines (data not shown). Furthermore, incubation of EGP2-positive tumor cells with a scFv:sTRAIL fusion protein containing a scFv of irrelevant target specificity did not induce apoptosis (Fig.3D). Apoptosis induction by scFvC54:sTRAIL was abrogated by co-incubation with TRAIL neutralizing MAb 2E5 (Fig.3C), which was observed for all cell lines tested (data not shown).

Apoptotic features induced by scFvC54:sTRAIL

Apoptotic features induced by scFvC54:sTRAIL were analyzed at elapsed time points of 1, 2, 3, 6, 12, and 24 h. For the initiator caspase-8 and the effector caspase-3 the following pattern was observed; activation was detectable after 3 h, which increased up to 6 h (Fig.4A). Activation levels of both caspase-8 and -3 decreased after 12 h. After 24 h no active caspase-8 or -3 could be detected. Increasing levels of active caspase-3 corresponded closely with declining DFF levels after 3 h and 6 h, with no DFF detectable after 12 h and 24 h (Fig.4A). Co-incubation of SW948 cells with scFvC54:sTRAIL and MAb MOC31 or MAb 2E5 completely inhibited apoptosis induction (Fig.4B, lanes 3 and 4, respectively). Treatment with culture medium containing no scFvC54:sTRAIL did not result in any of the apoptotic features analyzed (Fig.4B, lane 1).

TRAIL-R2 activation by scFvC54:sTRAIL

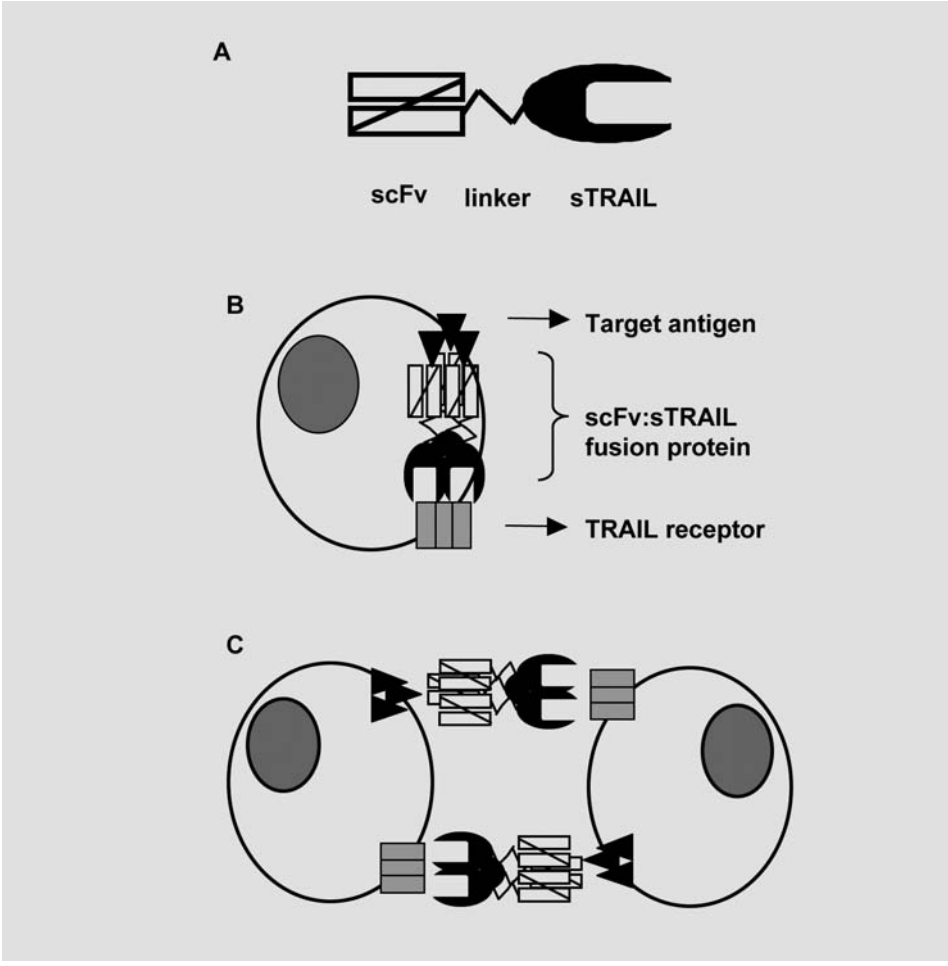
Jurkat cells (EGP2 neg.; TRAIL-R1 neg.; TRAIL-R2 pos.) were used to assess the specific activation of TRAIL-R2 by scFvC54:sTRAIL (Fig.5A). Jurkat cells proved to be insensitive to incubation with scFvC54:sTRAIL even for prolonged periods of time (Fig.5B and C; 6 h, 16 h). However, when Jurkat cells were incubated with scFvC54:sTRAIL in the presence of a multimeric form of the extracellular domain of EGP2 (sEGP2), strong induction of apoptosis was observed (Fig.5B and C; 6 h and 16 h). When increasing amounts of multimeric sEGP2 were added in the presence of a fixed concentration scFvC54:sTRAIL (350ng/ml), apoptosis was induced in a dose-dependent manner (Fig.5D). Jurkat cells cultured in the presence of the highest concentration multimeric sEGP2 alone showed no

signs of apoptosis (data not shown). Furthermore, only treatment with scFvC54:sTRAIL secondarily cross-linked with sEGP2 resulted in the activation of caspase-3 (Fig.5E) and complete DNA fragmentation after 16 h (Fig.5F).

Solution behavior of scFvC54:sTRAIL

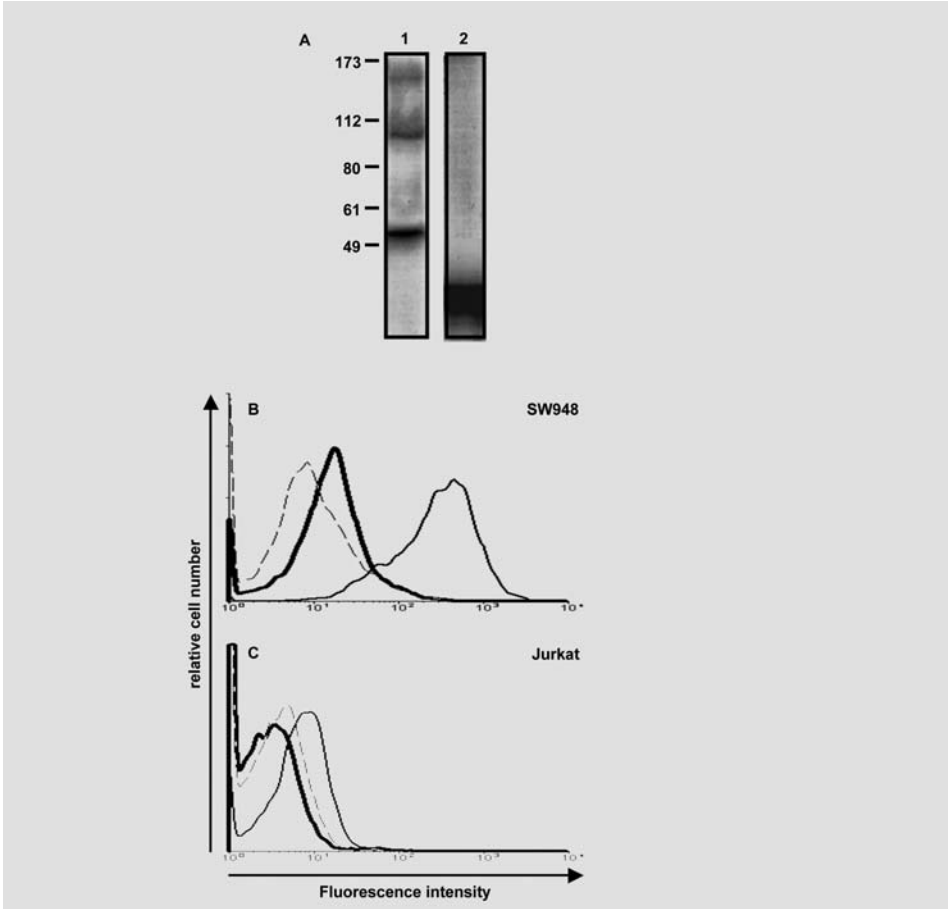
Supernatant containing scFvC54:sTRAIL was subjected to size exclusion (SE) FPLC. As indicated in Figure 6A, apoptosis induction of the TRAIL sensitive cell line SW948 was restricted to the individual samples collected after 95 to 115 minutes. TRAIL ELISA subsequently confirmed that only these fractions contained scFvC54:sTRAIL (Fig.6B). The elution peak of scFvC54:sTRAIL corresponded to a MW of approximately 160 kDa, which is in close proximity of the 154 kDa calculated for trimeric scFvC54:sTRAIL.

Figure 1



A: *Schematic presentation of a monomeric scFv:sTRAIL fusion protein in which a recombinant antibody fragment (scFv) is genetically fused to human sTRAIL via a linker sequence of 26 amino acid residues.*
B: *Target cell-restricted cross-linking of TRAIL receptors by a scFv:sTRAIL fusion protein. In principle, by binding to the target antigen (triangle), scFv:sTRAIL can cross-link agonistic TRAIL receptors and induce apoptosis in a monocellular fashion.*
C: *Additionally, scFv:sTRAIL can induce apoptosis in a bi-cellular fashion in which specific binding to one cell results in the cross-linking of TRAIL-receptors on a neighboring tumor cell. Figure adapted from Jung et al [42].*

Figure 2

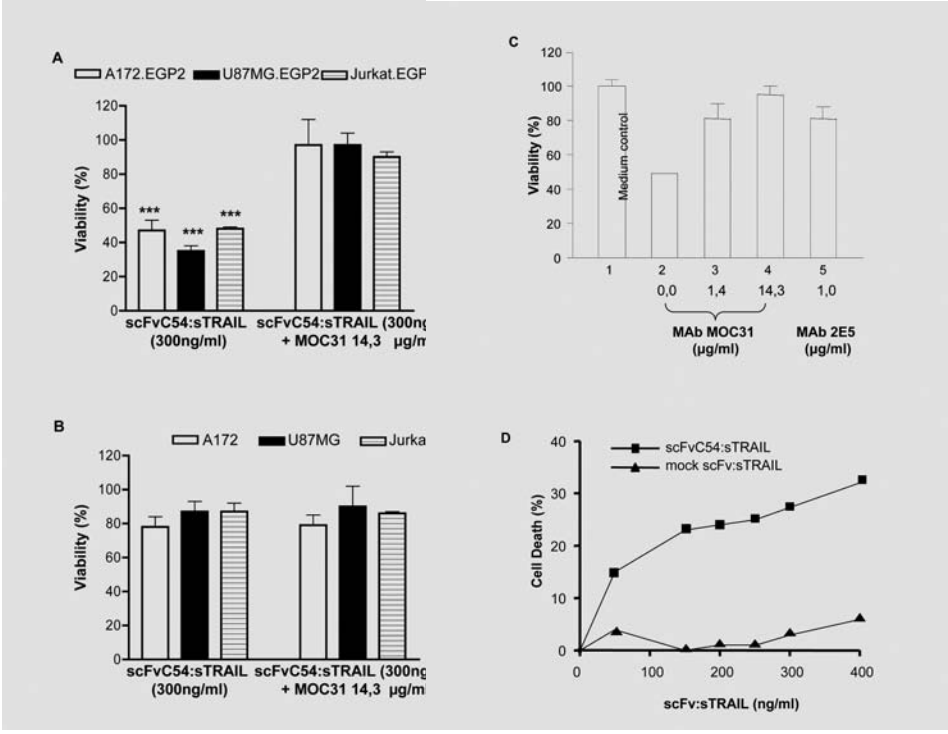


A: *Immunoblot analysis of scFvC54:sTRAIL. Lane 1, Under non-reducing conditions without sample boiling, scFvC54:sTRAIL was detected as monomers, dimers, and trimers (52, 102, and 154 kDa, respectively). Lane 2; Flag-tagged sTRAIL was detected as a monomer of 25 kDa (SDS-PAGE performed under reducing conditions with sample boiling). The relative positions of the M.W. markers are indicated by dashes.*

B: *Flow cytometric analysis of target antigen specific binding by scFvC54:sTRAIL. scFvC54:sTRAIL shows strong and specific binding to the EGP2 positive tumor cell line SW948 (solid line), which could be specifically inhibited by pre-incubation with anti-EGP2 MAb MOC31 (dashed line). Unconditioned medium control level is shown as a bold line.*

C: *Flow cytometric analysis of scFvC54:sTRAIL binding to target antigen negative cells.scFvC54:sTRAIL shows minimal binding to EGP2-negative Jurkat cells (solid line), which could be specifically blocked by pre-incubation with TRAIL-blocking MAb 2E5 (dashed line). Unconditioned medium control level is shown in bold. Binding of scFvC54:sTRAIL to other EGP2-negative cell lines was below detectable levels (data not shown).*

Figure 3



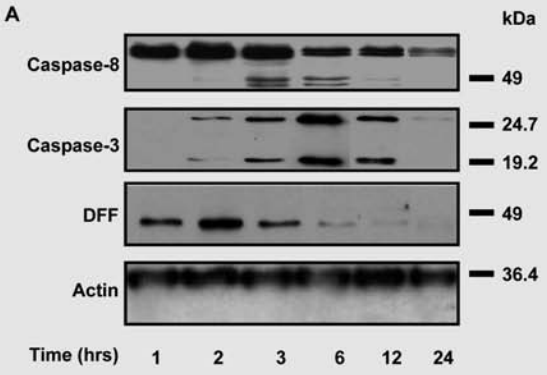
A: *Target antigen restricted apoptosis induction by scFvC54:sTRAIL. scFvC54:sTRAIL treatment of the EGP2-transduced cell lines A172.EGP2, U87MG.EGP2, and Jurkat.EGP2 resulted in target antigen restricted apoptosis induction (measured by MTS assay and indicated by % cell viability) that could be specifically blocked by MAb MOC31 (p<0,0001). Mean values and SDs are representatives of three independent experiments.*

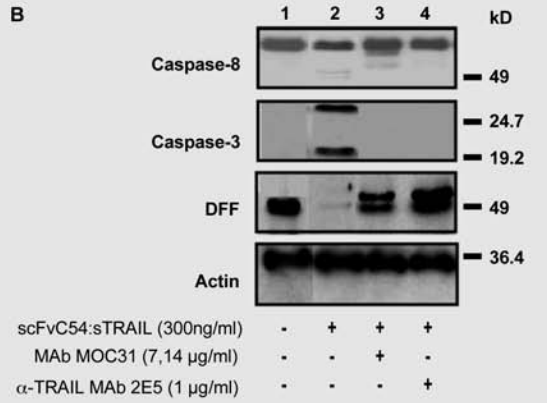
B: *Incubation of the EGP2 negative parental cell lines A172, U87MG and Jurkat with scFvC54:sTRAIL resulted in background levels of apoptosis (measured by MTS assay and indicated by % cell viability) that could not be blocked by pre-incubation with MAb MOC31. Mean values and SDs are representatives of three independent experiments.*

C: *Treatment of EGP2 positive SW948 cells with scFvC54:sTRAIL (300 ng/ml) strongly induced apoptosis (measured by MTS assay and indicated by % cell viability), which could be inhibited in a dose dependent manner by pre-incubation with increasing amounts of MAb MOC31 (bars 2, 3, & 4). Cell viability in medium without scFvC54:sTRAIL is shown in bar 1. Co-incubation with TRAIL neutralizing MAb 2E5 abrogated apoptosis induction (bar 5). Incubation with MAb MOC31 or MAb 2E5 alone had no effect on cell viability (data not shown). Mean values and SDs are representatives of three independent experiments.*

D: *Jurkat.EGP2 treated with increasing concentrations of a scFv:sTRAIL fusion protein containing a scFv of irrelevant target specificity (triangle) only showed background level of apoptosis induction. Treatment with increasing concentrations of scFvC54:sTRAIL (square) resulted in a dose dependent induction of apoptosis. Parental Jurkat cells were not sensitive to apoptosis induction by scFvC54:sTRAIL (data not shown).*

Figure 4

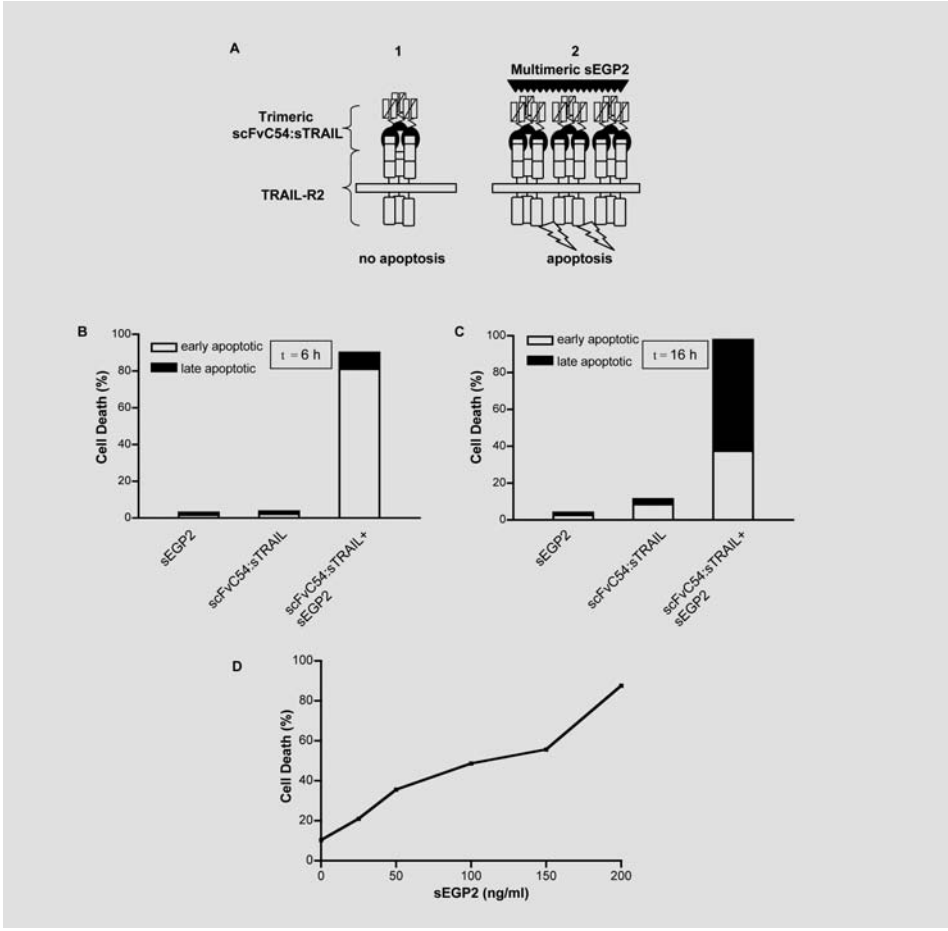




A: *Immunoblot analysis of intracellular apoptotic features induced by scFvC54:sTRAIL (300 ng/ml) in SW948 cells. Activation of initiator caspase-8 (43 kDa protein band) and effector caspase-3 (29 kDa protein band) was detectable after 3 h of treatment. Maximum activation levels were reached after 6 h, which decreased after 12 h, and were undetectable after 24 h of treatment. A marked decrease of the caspase-3 substrate DFF (45 kDa protein band) was observed after 3h treatment. No intact DFF was detectable after 24 h. Actin levels were analysed to confirm identical protein loading for all lanes.*

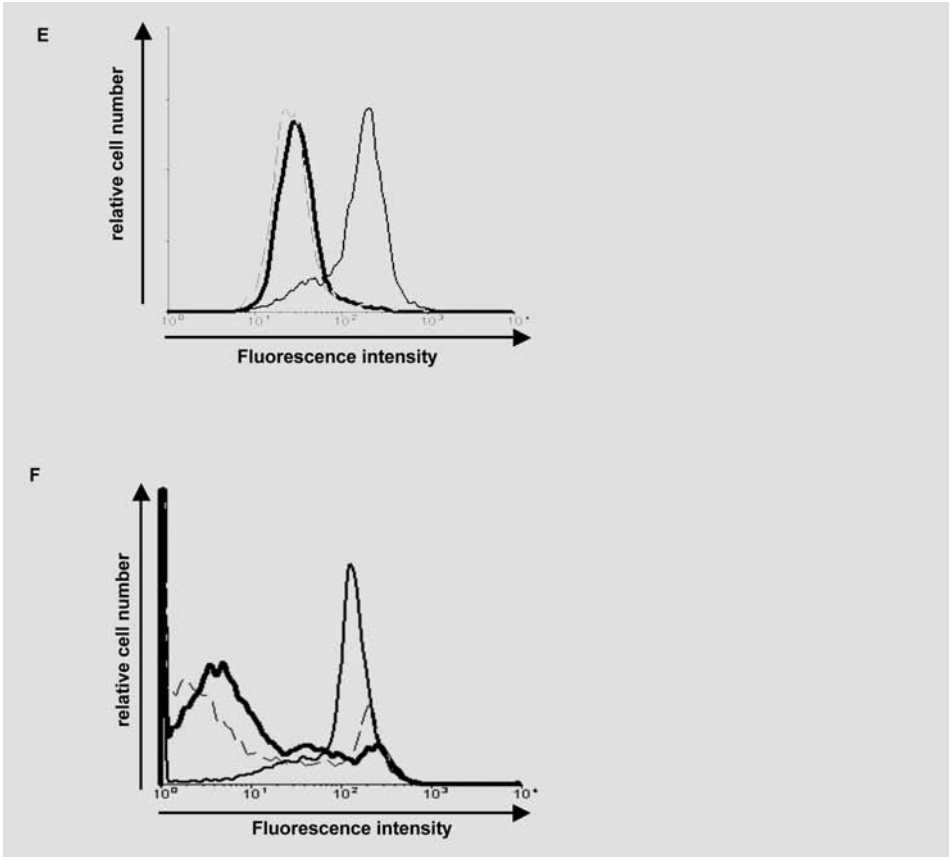
B: *Immunoblot analysis of target antigen restricted apoptosis induction after 12 h treatment with scFvC54:sTRAIL (300 ng/ml). Lane 1, no signs of apoptosis in the absence of scFvC54:sTRAIL; lane 2, treatment with scFvC54:sTRAIL results in cleavage of caspase-8, caspase-3, and complete degradation of DFF; lanes 3 and 4, pre-treatment with MAbs MOC31(7,4 mg/ml) or 2E5 (1 mg/ml) inhibits cleavage of caspase-8, caspase-3, and abrogates degradation of DFF. Note: In lanes 3 and 4, the addition of the murine MAbs resulted in the appearance of an additional antibody-derived protein band detected by the rabbit-anti-mouse HRPO-conjugated antibody.*

Figure 5



A: *Schematic representation of treatment of Jurkat cells (TRAIL-R1 neg ,TRAIL-R2 pos) with (1) scFvC54:sTRAIL alone and (2) scFvC54:sTRAIL that is secondarily cross-linked with a multimeric form of the extracellular domain of EGP2 (sEGP2).*
B,C: *AnnexinV-FITC/PI staining identified early apoptotic cells with phosphatidyl serine exposure on the outer cell membrane and late apoptotic AnnexinV-FITC/PI double positive cells after treatment of Jurkat cells with scFvC54:sTRAIL (300 ng/ml) in the absence or presence of sEGP2 (200 ng/ml). Jurkat cells treated with scFvC54:sTRAIL secondarily cross-linked with sEGP2 showed a dramatic increase in early (white part of bar) and late apoptotic cells (black part of bar) after 6 h (B) and 16 h (C). Incubation with scFvC54:sTRAIL or sEGP2 alone had no effect on apoptosis induction.*
D: *Addition of increasing amounts of multimeric sEGP2 dose-dependently increased the level of apoptosis induction by a fixed concentration of scFvC54:sTRAIL (350ng/ml) in Jurkat cells. In the absence of multimeric sEGP2 only background level of apoptosis was observed. Increasing amounts of sEGP2 resulted in a dose dependent 5 fold increase in apoptosis induction at 200 ng sEGP2.*

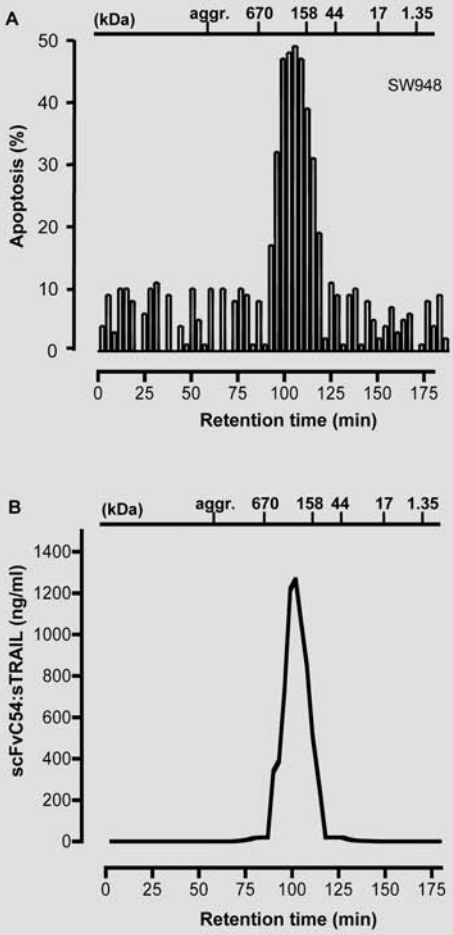
Figure 5



E: *Flow cytometric analysis of caspase activation in Jurkat cells by sEGP2 cross-linked scFvC54:sTRAIL. Caspase activation by scFvC54:sTRAIL (300 ng/ml) in the presence or absence of multimeric sEGP2 (200 ng/ml) was analysed using the caspscreen flow cytometry apoptosis detection kit that detects cleavage of the non-fluorescent substrate (aspartyl)2-Rhodamine 110 (D2R) into fluorescent Rhodamine 110 by activated caspases. Treatment of Jurkat cells with scFvC54:sTRAIL secondarily cross-linked with sEGP2 resulted in a marked increase in fluorescence intensity (solid line). Treatment with scFvC54:sTRAIL alone (dashed line) or with unconditioned medium (bold line) did not result in caspase activation.*

F: *Flowcytometric analysis of DNA fragmentation in Jurkat cells by sEGP2 cross-linked scFvC54:sTRAIL. DNA fragmentation by scFvC54:sTRAIL (300 ng/ml) in the presence or absence of multimeric sEGP2 (200 ng/ml) was analysed using MAb F7-26 that specifically binds to ssDNA that has been fragmented by the apoptotic process (see material and methods). Treatment of Jurkat cells with scFvC54:sTRAIL secondarily cross-linked with sEGP2 for 16 h resulted in a marked increase in fluorescence intensity (solid line), indicating strong and complete DNA fragmentation (solid line). Treatment with scFvC54:sTRAIL alone (dashed line) or with unconditioned medium (bold line) did not result in apoptotic DNA fragmentation.*

Figure 6



Solution behavior and apoptosis inducing activity of scFvC54:sTRAIL after size exclusion (SE) FPLC. Medium containing scFvC54:sTRAIL (3,44 mg/ml) was subjected to SE-FPLC using a HiLoad Superose 200 column. Individual samples collected at 3-minute intervals were analysed for (A) their capacity to induce apoptosis using the TRAIL sensitive cell line SW948 and (B) for scFvC54:sTRAIL content using a sensitive TRAIL specific ELISA. Apoptosis induction was restricted to samples collected after 95 min. to 115 min. TRAIL ELISA confirmed that only these fractions contained scFvC54:sTRAIL. The elution peak of scFvC54:sTRAIL corresponded to a molecular weight of approximately 160 kDa, which is in close proximity of 154 kDa calculated for trimeric scFvC54:sTRAIL. The retention time of the calibration standards indicated are: thyroglobulin, 670kDa, 85 min; g-globulin, 158kDa, 106 min; ovalbumin, 44kDa, 124 min; myoglobin, 17kDa, 138 min; vitamin B-12, 1.35kDa, 161 min. High molecular weight protein aggregates (aggr.) present in the calibration standard eluted after 58 min.

Discussion

Human sTRAIL appears to be a promising new anti-cancer agent. However, the widespread expression of TRAIL receptors throughout the human body and the recently reported possible TRAIL-related toxicity towards certain normal cells, at least of certain recombinant forms of this cytokine, might hamper its clinical development. Augmentation of the therapeutic value of sTRAIL can be achieved by increasing its tumor selective binding properties through the genetic fusion to a tumor-selective antibody fragment[23]. Here we demonstrated that specific targeting of sTRAIL to EGP2-positive cancer cells can be attained by the scFvC54:sTRAIL fusion protein, in which the carcinoma specific antibody fragment scFvC54 is genetically linked to the N-terminus of human sTRAIL. The high affinity scFvC54 domain specifically recognizes EGP2, an established cell surface target antigen that is highly overexpressed on a variety of human carcinomas[39,40,41]. Specific binding of scFvC54:sTRAIL to EGP2-positive tumor cells was readily demonstrated by flow cytometry. Pre-incubation with a competing anti-EGP2 MAb MOC31 selectively blocked the binding in a dose-dependent manner. Binding to EGP2-negative cell lines was below detectable levels, except for Jurkat cells to which a weak extracellular binding was observed that could be blocked by a TRAIL-neutralizing MAb. Together this demonstrated that the scFvC54 targeting domain strongly enhanced the tumor selective binding of scFvC54:sTRAIL to EGP2-positive tumor cells only.

FACS data further indicated that EGP2-specific binding converted soluble scFvC54:sTRAIL into an artificial membrane bound form of TRAIL. Since the number of EGP2 target molecules greatly exceeded that of the TRAIL-receptors on the same cell, a surplus of sTRAIL domains was available for subsequent cross-linking of agonistic TRAIL receptors on neighboring tumor cells. When EGP2-positive tumor cells (Jurkat.EGP2, A172.EGP2, and U87MG.EGP2) were subjected to treatment with scFvC54:sTRAIL, an efficient induction of apoptosis was observed, which in all cases could be inhibited by pre-incubation with MAb MOC31 or a TRAIL-neutralizing MAb. This indicated that scFvC54:sTRAIL efficiently induced bi- or multi-cellular reciprocal fratricide apoptosis in a target antigen restricted fashion. This principle has been previously described in a study on the specific targeting of Fas-mediated apoptosis induction[42]. In this study by Jung et al. a bi-specific antibody fragment was used, comprising a non-activating monomeric CD95 antibody hybridized to a second monomeric antibody targeting CD20 present on the same tumor cell.

Recently, it was shown that TRAIL receptors 1 and 2 have quite distinct cross-linking requirements for the initiation of apoptosis[22]. TRAIL-R2 appears to signal apoptosis only after efficient receptor cross-linking by either native memTRAIL, aggregated sTRAIL variants, or by sTRAIL preparations secondarily cross-linked by antibodies. Apoptosis signaling by TRAIL-R1 appears to be relatively independent of the receptor cross-linking characteristics of a particular form of sTRAIL. Furthermore, it was shown that TRAIL-R2 had superior binding affinity for TRAIL, resulting in predominant binding of sTRAIL to TRAIL-R2 over TRAIL-R1[10].

To analyze the TRAIL receptor cross-linking effects of scFvC54:sTRAIL, we exploited Jurkat cells, a cell line that expresses TRAIL-R2 but no detectable levels of TRAIL-R1, resistant to relatively high concentrations of non-aggregated sTRAIL. When Jurkat.EGP2 transfectant cells were subjected to scFvC54:sTRAIL treatment, efficient induction of apoptosis of up to 60% was achieved, indicating that target antigen restricted apoptosis induction in these cells is initiated via TRAIL-R2 cross-linking. Furthermore, when parental Jurkat cells were subjected to a fixed concentration of scFvC54:sTRAIL, in the presence of increasing amounts of a multimeric form of sEGP2, a dramatic increase in apoptosis induction was observed, which directly correlated to the concentration of cross-linking multimeric sEGP2 added (see Fig. 5D). From this it can be concluded that the target antigen restricted apoptosis inducing capacity of scFvC54:sTRAIL is directly proportional to the degree of TRAIL-R2 receptor cross-linking. Moreover, this data indicates that scFvC54:sTRAIL can overcome TRAIL-resistance related to the differential expression of TRAIL-R2 over TRAIL-R1 as is observed for many different TRAIL resistant cell lines. The parental cell lines Jurkat, A172 and U87MG, used in the present study, exemplify the preferential expression of TRAIL-R2 over TRAIL-R1 and the subsequent insensitivity to treatment with scFvC54:sTRAIL. Interestingly, SW948 cells (EGP2-positive colon carcinoma cells) expressing both TRAIL-R1 and TRAIL-R2, were almost completely rescued from apoptosis by pre-incubation with blocking MAb MOC31 (Fig. 3C). This implies that, even in the presence of TRAIL-R1, scFvC54:sTRAIL-mediated apoptosis appears to be predominantly initiated by TRAIL-R2. Although not formally proven here, it appears that this possibly is the result of the preferential cross-linking capacity of scFvC54:sTRAIL for the high affinity TRAIL-R2 receptor over TRAIL-R1, which binds TRAIL with lower affinity[10].

The current data independently corroborate with the results previously published by Wajant et al.[23]. Wajant et al demonstrated that the restricted signaling capacity of sTRAIL could be converted into a TRAIL-R2 stimulating ligand after genetic fusion to a scFv antibody fragment specific for the tumor stroma marker Fibroblast Activation Protein (FAP).

Recently, several papers reported on the apoptosis inducting potential of certain recombinant sTRAIL preparations towards primary human cells such as normal human hepatocytes43, keratinocytes44, prostrate epithelial cells[45], and brain tissue[46]. It has been suggested that this potential toxicity is related to high molecular weight sTRAIL aggregates present in certain sTRAIL preparations[47]. Purified his-tagged sTRAIL, refolded from bacterial expression systems, appeared to contain TRAIL-aggregates that might be directly responsible for the hepatocyte toxicity observed for this particular preparation. Thus, the production of non-aggregated sTRAIL derivatives appears to be important in order to avoid organ-specific or systemic toxicity.

In the present study, we aimed at producing biologically active and correctly folded scFvC54:sTRAIL by directing it through the endoplasmatic reticulum of eukaryotic CHO-K1 cells, thus taking advantage of the associated stringent quality control mechanisms

ensuring that only correctly folded and non-aggregated fusion protein is secreted into the culture medium. The anti-FAP scFv:sTRAIL fusion protein, described by Wajant et al23, was transiently expressed from transfected COS-7 cells. However, transient expression systems are intrinsically subject to batch-to-batch variations and in their study no data was presented on molecular weight, or solution behavior of the produced fusion protein.

Anticipating its clinical potential, we produced scFvC54:sTRAIL in Chinese Hamster Ovary (CHO) cells, a currently favored host cell type for the production of therapeutic recombinant proteins. The Glutamine Synthetase system was used to generate a CHO production cell line clone 70C1 that stably secreted high levels of scFvC54:sTRAIL in to the medium and allowed for large scale production. To assess for the presence of scFvC54:sTRAIL aggregates in the production medium, we exploited the high sensitivity of Jurkat cells to such aggregates. When Jurkat cells were exposed to prolonged incubation with medium containing up to 3,4 µg/ml scFvC54:sTRAIL, no signs of apoptosis or toxicity were observed.

Subsequent analysis of the solution behavior of scFvC54:sTRAIL by size exclusion (SE) FPLC produced a single TRAIL activity peak corresponding to a molecular weight of approximately 160 kDa (Fig. 6). This is in close proximity of the 154 kDa that can be calculated for scFvC54:sTRAIL trimers. From this it can be concluded that scFvC54:sTRAIL is produced as soluble homogeneous trimers with no or only minimal aggregate formation.

Stable trimeric scFvC54:sTRAIL contains three identical scFvC54 domains, which potentially enhances binding to EGP2-positive cells by the associated avidity effect. Enhanced avidity has been shown to be beneficial for *in vivo* tumor targeting in many antibody-based therapeutic strategies[48,49]. In our experiments, equimolar concentrations of the bivalent high affinity (<10nm) anti-EGP2 MAb MOC31 were needed to inhibit binding of trimeric scFvC54:sTRAIL to EGP2-positive cells, indicating that both molecules are at least of similar affinity. We designed scFvC54:sTRAIL to contain a spacer of 26 amino acids between the scFvC54 domain and sTRAIL. The length and flexibility of this spacer, containing a SSGSG hydrophilic region, were employed to prevent steric hindrance during simultaneous binding of trimeric scFvC54:sTRAIL to three EGP2 molecules and one (trimeric) TRAIL-R molecule.

In conclusion, to fully exploit the therapeutic potential of sTRAIL, characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. Firstly, the wide spread expression of the various TRAIL receptors throughout the human body; secondly, the differential binding affinities and cross-linking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2; and thirdly, the solution behavior of particular sTRAIL preparations. The fusion protein scFvC54:sTRAIL complies with these notions and is the first example of a sTRAIL variant with enhanced tumor selective apoptosis induction

towards EGP2-positive tumor cells. The favorable characteristics of scFvC54:sTRAIL potentially reduce the amount of TRAIL required for anti-tumor activity and may thereby reduce the risk of potential toxicity associated with conventional non-targeting sTRAIL preparations.

Nevertheless, it cannot be completely excluded that even scFv:sTRAIL fusions proteins such as scFvC54:sTRAIL, described here, and the anti-FAP scFv:sTRAIL fusion protein, described by Wajant et al, might exert certain toxic effects towards normal human tissues expressing the respective antigen. In normal epithelia EGP2 is shielded by an intact basal membrane[26,27], thereby reducing the risk of targeting scFvC54:sTRAIL to non-malignant epithelial tissue. Toxicity of scFvC54:sTRAIL can, in part, be analyzed by using the human EGP2 transgenic mouse[50] model that was recently generated in our lab. In this transgenic mouse, human EGP2 expression exhibits an authentic epithelial expression pattern that is comparable to the human situation. Unfortunately, human FAP-transgenic animal models are currently not available, which might potentially hamper the further pre-clinical evaluation of the anti-FAP scFv:sTRAIL fusion protein.

From the present *in vitro* study we conclude that targeting of sTRAIL to the abundant tumor associated target antigen EGP2, and possibly to a number of other known surface-expressed tumor antigens like the Epidermal Growth Factor Receptor, is a promising anti-tumor strategy that might be useful in clinical application for various human carcinomas.

Acknowledgements: This work was supported by a grant from the Dutch Cancer Society; project grant nr. RUG 2002-2668. We thank Geert Mesander, Linda van Genne, and Jelleke Dokter-Fokkens for their excellent technical assistance.

References

1. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995, 3, 673-682
2. Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A., Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J.Biol.Chem. 1996, 271, 12687-12690
3. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H., Safety and antitumor activity of recombinant soluble Apo2 ligand. J.Clin.Invest 1999, 104, 155-162
4. Roth, W., Isenmann, S., Naumann, U., Kugler, S., Bahr, M., Dichgans, J., Ashkenazi, A., and Weller, M., Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem.Biophys.Res.Commun. 1999, 265, 479-483
5. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H., Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat.Med. 1999, 5, 157-163
6. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M., The receptor for the cytotoxic ligand TRAIL. Science 1997, 111-113
7. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M., An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 1997, 277, 815-818
8. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T., TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. 1997, 16, 5386-5397
9. Griffith, T. S. and Lynch, D. H., TRAIL: a molecule with multiple receptors and control mechanisms. Curr.Opin.Immunol. 1998, 10, 559-563
10. Truneh, A., Sharma, S., Silverman, C., Khandekar, S., Reddy, M. P., Deen, K. C., McLaughlin, M. M., Srinivasula, S. M., Livi, G. P., Marshall, L. A., Alnemri, E. S., Williams, W. V., and Doyle, M. L., Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. J.Biol.Chem. 2000, 275, 23319-23325
11. MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S., Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J.Biol.Chem. 1997, 272, 25417-25420
12. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A., A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr.Biol. 1997, 7, 1003-1006
13. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G., The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity. 1997, 7, 813-820
14. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E., Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBOJ. 1995, 14, 5579-5588
15. Peter, M. E., The TRAIL DISCussion: It is FADD and caspase-8! Cell Death.Differ. 2000, 7, 759-760
16. Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A., Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors

4 and 5. Immunity. 2000, 12, 611-620
17. Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H., and Walczak, H., FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity. 2000, 12, 599-609
18. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M., FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995, 81, 505-512
19. Sprick, M. R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M. A., and Walczak, H., Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J. 2002, 21, 4520-4530
20. Kischkel, F. C., Lawrence, D. A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D., and Ashkenazi, A., Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J.Biol.Chem. 2001, 276, 46639-46646
21. Wang, J., Chun, H. J., Wong, W., Spencer, D. M., and Lenardo, M. J., Caspase-10 is an initiator caspase in death receptor signaling. Proc.Natl.Acad.Sci.U.S.A 2001, 98, 13884-13888
22. Muhlenbeck, F., Schneider, P., Bodmer, J. L., Schwenzer, R., Hauser, A., Schubert, G., Scheurich, P., Moosmayer, D., Tschopp, J., and Wajant, H., The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J.Biol.Chem. 2000, 275, 32208-32213
23. Wajant, H., Moosmayer, D., Wuest, T., Bartke, T., Gerlach, E., Schonherr, U., Peters, N., Scheurich, P., and Pfizenmaier, K., Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 2001, 20, 4101-4106
24. Balzar, M., Winter, M. J., de Boer, C. J., and Litvinov, S. V., The biology of the 17-1A antigen (Ep-CAM). J.Mol.Med. 1999, 77, 699-712
25. Moldenhauer, G., Momburg, F., Moller, P., Schwartz, R., and Hammerling, G. J., Epithelium-specific surface glycoprotein of Mr 34,000 is a widely distributed human carcinoma marker. Br.J.Cancer 1987, 56, 714-721
26. Bumol, T. F., Marder, P., DeHerdt, S. V., Borowitz, M. J., and Apelgren, L. D., Characterization of the human tumor and normal tissue reactivity of the KS1/4 monoclonal antibody. Hybridoma 1988, 7, 407-415
27. Momburg, F., Moldenhauer, G., Hammerling, G. J., and Moller, P., Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res. 1987, 47, 2883-2891
28. Ragnhammar, P., Fagerberg, J., Frodin, J. E., Hjelm, A. L., Lindemalm, C., Magnusson, I., Masucci, G., and Mellstedt, H., Effect of monoclonal antibody 17-1A and GM-CSF in patients with advanced colorectal carcinoma--long-lasting, complete remissions can be induced. Int.J.Cancer 1993, 53, 751-758
29. Ragnhammar, P., Magnusson, I., Masucci, G., and Mellstedt, H., The therapeutic use of the unconjugated monoclonal antibodies (MAb) 17-1A in combination with GM-CSF in the treatment of colorectal carcinoma (CRC). Med.Oncol.Tumor Pharmacother. 1993, 10, 61-70
30. Riethmuller, G., Holz, E., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Funke, I., Pichlmaier, H., Hirche, H., Buggisch, P., Witte, J., and Pichlmayr, R., Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. J.Clin.Oncol. 1998, 16, 1788-1794
31. Helfrich, W., Koning, P. W., The, T. H., and de Leij, L., Epitope mapping of SCLC-cluster-2 MAbs and generation of antibodies directed against new EGP2 epitopes. Int.J.Cancer Suppl 1994, 8, 64-69

32. Huls, G. A., Heijnen, I. A., Cuomo, M. E., Koningsberger, J. C., Wiegman, L., Boel, E., van der Vuurst de Vries AR, Loyson, S. A., Helfrich, W., Berge Henegouwen, G. P., van Meijer, M., de Kruif, J., and Logtenberg, T., A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat.Biotechnol. 1999, 17, 276-281
33. Helfrich, W., Van Geel, M., The, T. H., and de Leij, L., Detection of a putative 30-kDa ligand of the cluster-2 antigen. Int.J.Cancer Suppl 1994, 8, 70-75
34. Schneider, P., Thome, M., Burns, K., Bodmer, J. L., Hofmann, K., Kataoka, T., Holler, N., and Tschopp, J., TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity. 1997, 7, 831-836
35. Kinsella, T. M. and Nolan, G. P., Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum.Gene Ther. 1996, 7, 1405-1413
36. Helfrich, W., Haisma, H. J., Magdolen, V., Luther, T., Bom, V. J., Westra, J., van der, Hoeven R., Kroesen, B. J., Molema, G., and de Leij, L., A rapid and versatile method for harnessing scFv antibody fragments with various biological effector functions. J.Immunol.Methods 2000, 237, 131-145
37. Cockett, M. I., Bebbington, C. R., and Yarranton, G. T., High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. Biotechnology (N.Y.) 1990, 8, 662-667
38. Bodmer, J. L., Meier, P., Tschopp, J., and Schneider, P., Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. J.Biol.Chem. 2000, 275, 20632-20637,
39. Poczatek, R. B., Myers, R. B., Manne, U., Oelschlager, D. K., Weiss, H. L., Bostwick, D. G., and Grizzle, W. E., Ep-Cam levels in prostatic adenocarcinoma and prostatic intraepithelial neoplasia. J.Urol. 1999, 162, 1462-1466
40. Piyathilake, C. J., Frost, A. R., Weiss, H., Manne, U., Heimburger, D. C., and Grizzle, W. E., The expression of Ep-CAM (17-1A) in squamous cell cancers of the lung. Hum.Pathol. 2000, 31, 482-487
41. Ogura, E., Senzaki, H., Yoshizawa, K., Hioki, K., and Tsubura, A., Immunohistochemical localization of epithelial glycoprotein EGP2 and carcinoembryonic antigen in normal colonic mucosa and colorectal tumors. Anticancer Res. 1998, 18, 3669-3675
42. Jung, G., Grosse-Hovest, L., Krammer, P. H., and Rammensee, H. G., Target cell-restricted triggering of the CD95 (APO-1/Fas) death receptor with bispecific antibody fragments. Cancer Res. 2001, 61, 1846-1848
43. Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Billiar, T. R., and Strom, S. C., Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat.Med. 2000, 6, 564-567
44. Leverkus, M., Neumann, M., Mengling, T., Rauch, C. T., Brocker, E. B., Krammer, P. H., and Walczak, H., Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res. 2000, 60, 553-559
45. Nesterov, A., Ivashchenko, Y., and Kraft, A. S., Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells. Oncogene 2002, 21, 1135-1140
46. Nitsch, R., Bechmann, I., Deisz, R. A., Haas, D., Lehmann, T. N., Wendling, U., and Zipp, F., Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). Lancet 2000, 356, 827-828
47. Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., Hooley, J., Sherwood, S., Pai, R., Leung, S., Khan, L., Gliniak, B., Bussiere, J., Smith, C. A., Strom, S. S., Kelley, S., Fox, J. A., Thomas, D., and Ashkenazi, A., Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat.Med. 2001, 7, 383-385
48. Power, B. E. and Hudson, P. J., Synthesis of high avidity antibody fragments (scFv multimers) for cancer imaging. J.Immunol.Methods 2000, 242, 193-204

49. Kortt, A. A., Dolezal, O., Power, B. E., and Hudson, P. J., Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting. Biomol.Eng 2001, 18, 95-108
50. McLaughlin, P. M., Harmsen, M. C., Dokter, W. H., Kroesen, B. J., van der, Molen H., Brinker, M. G., Hollema, H., Ruiters, M. H., Buys, C. H., and de Leij, L. F., The epithelial glycoprotein 2 (EGP2) promoter-driven epithelial-specific expression of EGP2 in transgenic mice: a new model to study carcinoma-directed immunotherapy. Cancer Res. 2001, 61, 4105-4111

Chapter 5

The efficacy of alginate encapsulated CHO-K1 single chain-TRAIL producer cells in the treatment of brain tumors

Jos MA Kuijlen[1], Bart J de Haan[2], Wijnand Helfrich[2], Jan-Freark de Boer[2], Douwe Samplonius[2], Jan Jakob A Mooij[2], Paul de Vos[2]

[1]Departments of Neurosurgery
[2]and Pathology and Laboratory Medicine
University Medical Center Groningen, The Netherlands

J Neurooncol. 2006;78(1):31-9

Abstract

Object: Patients with astrocytic tumors in the central nervous system (CNS) have low survival rates despite surgery and radiotherapy. Innovative therapies and strategies must be developed to prolong survival of these patients. The alginate microencapsulation method, used to continuously release a certain cytotoxic agent in the vicinity of the tumor, is such a novel therapeutic strategy. The biological functionality of the apoptosis inducing scFv425:sTRAIL protein, which was released through the microencapsulation method, was studied in vitro. Analysis of the intracerebral biocompatibility of alginate capsules was performed by implantation of empty alginate capsules in the brain of mice.

Method: Chinese Hamster Ovary cells (CHO-K1) were recombinantly engineered to produce the single chain anti-EGFR-sTRAIL protein (scFv425:sTRAIL). The CHO-K1 producer cells were encapsulated in an alginate capsule with a semi-permeable membrane through which the scFv425:sTRAIL protein could be released.

Results: In vitro studies show maintained biological functionality of the released scFv425:sTRAIL protein. There was no immunological tissue response detectable after intracerebral implantation of the alginate capsules in mice brains.

Conclusion: Biological functionality of the produced scFv425:sTRAIL protein is maintained and intracerebral biocompatibility of the capsules is warranted. Alginate encapsulation of CHO-K1 - scFv425:sTRAIL - producer cells and subsequently their intracerebral implantation is technically feasible. This study justifies further in vivo experiments.

Introduction

Central nervous system (CNS) malignancies are characterized by low survival rates despite their treatment with conventional therapies such as surgery and radiotherapy. New therapies have to be developed to prolong the overall survival of patients with these CNS malignancies.

Soluble TRAIL (sTRAIL) is a ligand, which can induce apoptosis in tumor cells after binding to the death inducing receptors TRAIL-R1 and TRAIL-R2. TRAIL has sequence homology with TNF and FasL [1]. It has been shown that TRAIL induces cell death in various cancer cells including highly malignant glioma cell lines [1-4]. The advantage of TRAIL over TNF and FasL administration is the fact that it is non-toxic to normal cells [5-8]. A disadvantage of sTRAIL is that it has no specificity for tumor tissue alone. Binding to normal tissues, which also have TRAIL receptors, such as liver and bone marrow, results in a lower availability of sTRAIL for tumor cells. It has been shown that sTRAIL predominantly binds to TRAIL-R1 in a non-cross-linked manner whereas TRAIL-R2 is only activated by cross-linked sTRAIL [9]. As TRAIL-R2 receptors are more expressed on cell membranes of malignant glioma cells than TRAIL-R1 [10] it is expected that activation of TRAIL-R2 receptors by cross-linked sTRAIL is of more importance in the induction of apoptosis in malignant cancer cells than activation of the TRAIL-R1 receptor.

To enhance specificity of sTRAIL for tumor cells and to optimize the apoptosis inducing capacity of the TRAIL-R2 receptor, a single chain:sTRAIL protein (scFv:sTRAIL) was developed.

In an earlier publication the development of a scFv:sTRAIL protein and the concept of targeting sTRAIL to tumor cells was described [11]. The author (WH) developed a recombinantly engineered scFv:sTRAIL protein with specificity for the epidermal growth factor receptor (EGFR), which is abundantly present on glioma cells. This protein is designated scFv425:sTRAIL and has many characteristics for successful application as an antineoplastic therapy in the treatment of patients with a glioblastoma multiforme tumor (GBM).

In order to eradicate tumor cells with scFv:sTRAIL proteins, several methods of administration to brain tumors can be applied. Such methods are intravenous, intra-arterial administration with osmotic blood-brain-barrier disruption and chronic intratumoral infusion (i.e. convection enhanced delivery). An alternative is the implantation of encapsulated (scFv:sTRAIL) producer cells in the vicinity of the tumor. Studies concerning the application of alginate encapsulated producer cells to cure neurodegenerative diseases and brain tumors have been shown to have some degree of success [12-17].

Up to now insufficient biocompatibility of the capsules and subsequent death of the encapsulated producer cells has hampered the success and clinical application of

the technology. Recent advances such as application of pure alginates with a defined composition has brought new insight in the factors determining the biocompatibility of the capsules.

In the present study, types of alginate capsules prepared of high guluronic (G)-acid or intermediate guluronic-acid were used. Intermediate guluronic-acid capsules are in contrast to high guluronic-acid capsules more flexible but less durable [18]. High-G alginates are more durable and are associated with less protruding cells but, as has been shown in other sides than the brain, less biocompatible [18,19].

In the present study, we test both types of alginate in a highly purified composition for their intracerebral biocompatibility and principle applicability. Since the type of alginate [18,19] influences the functionality of cell-types, we subsequently applied the two types of alginates for encapsulation of scFv425:sTRAIL protein producing Chinese Hamster Ovary producer cells. Analysis of the cell kinetics of the encapsulated CHO-K1 producer cells was performed. The supernatant, containing the scFv425:sTRAIL protein, which was released from the alginate capsules, was tested for its death inducing capacity.

Material and Methods

Design of the study

Alginate microcapsules were implanted through a burr hole in the brain of C57BL6-mice. Only highly purified alginates were applied in order to exclude that contaminating components were the cause of an inflammatory response. Capsules were prepared of alginates with either an intermediate guluronic acid (G) content (40% G) or a high G-content (> 50% G). The capsules were inspected before and after implantation in order to confirm that the majority of the capsules were intact. Three experimental groups were defined: group 1 consisted of 6 mice with an intracerebral implant of a single high-G capsule, group 2: 6 mice with an intracerebral implant of an intermediate-G capsule and group 3: a sham experiment with introduction of the needle in the absence of a capsule and infusion of KRH solution only. The capsules were retrieved at two weeks post implant to study the degree of overgrowth, i.e. a measure for the capsules biocompatibility.

In the subsequent *in vitro* study, CHO-K1 cells producing the single chain protein (scFv425:sTRAIL) were encapsulated in capsules prepared of intermediate-G or high-G alginate. Growth kinetics of the cells and the biological activity of the produced protein was tested at several time points up to 30 days after encapsulation.

Cell lines

The CHO-K1, A172 and SW948 cell lines were purchased from ATCC. Cells (CHO-K1, SW948) were cultured in flasks (75 cm^2 , Ventcap, Corning Inc, New York) containing RPMI-1640 (Gibco BRL, Life Technologies) and were supplemented with sodiumpyruvate, glutamine, gentamycine, fungizone and 14% FCS.

A172 (glioma) spheroids were prepared by seeding 8x105 cells in 4 ml of DMEM in 25 cm^2 flasks. The plates were coated with a 1% agar/DMEM solution to facilitate cell growth. Fresh medium was added every third day. Within 2 weeks spheroids were formed with a diameter of approximately 2 mm. Cells were stored at 37 ºC in humidified 5% CO_2 atmosphere. The growth medium of the encapsulated CHO-K1 producer cells was refreshed every third to fourth day. The culture flasks were changed once a week.

Construction of single chain (scFv):sTRAIL protein

The construction of a scFv:sTRAIL protein was described in detail elsewhere by one of the authors (WH) [11]. Eukaryotic expression plasmid pEE14scFv:sTRAIL was generated

for the rapid construction, evaluation, and stable expression of scFv:sTRAIL proteins in CHO-K1 cells. The procedure identified a recombinant CKO-K1 production cell line clone, that stable excreted scFv425:sTRAIL in the medium at a concentration of 2.07 µg/ml. Large scale production of scFv:sTRAIL protein was performed by culturing the clone in roller bottles (Greiner Bio-One GmbH, Frickenhausen, Germany) at 37ºC in serum free CHO-S SFM II suspension medium (Gibco, Life Technologies BV, Breda, The Netherlands) to a density of $0{,}5x10^6$ cells/ml, after which supernatant was harvested (1500xg; 10 min).

Microencapsulation

In the present study, we only applied highly purified alginate. The alginate production process has been described in detail elsewhere [20]. Intermediate-G and high-G sodium alginates were purchased from Kelco International, London, UK. Crude sodium alginate (Keltone LV, Kelco International, London, UK) was dissolved at 40C in a 1 mM sodium EGTA solution to a 1% solution for intermediate-G alginate and to a 0.25 % solution for high-G alginate under constant stirring. Subsequently these solutions were filtered to remove aggregates. The pH of the solution was lowered to pH 2 to gradually precipitate alginate to alginic acid. A washing procedure was performed to wash out non-precipitated contaminants. This was repeated three times. Subsequently, proteins were removed by extraction with chloroform/butanol, which was also repeated three times. Next, the alginic acid was brought in water and slowly dissolved. The alginate solution obtained was subjected to a chloroform/butanol extraction to remove inflammatory proteins, which can only be dissolved in chloroform/butanol at neutral pH. The last step was precipitation of the alginate with ethanol. Finally, the alginate was freeze-dried overnight. Endotoxin content of purified alginate samples was assessed by a commercial Limulus-lysate assay following the protocol of the E-toxate kit recommended by Sigma.

In the procedures to form capsules of intermediate-G alginate and high-G alginate, the physical stability was kept optimal by applying an alginate solution with the highest viscosity possible. This viscosity of alginate solutions is restricted by the sterilization process of alginates *(i.e.* 0.2 µm filtration), which requires that the solutions have a viscosity lower than 4cps.

Purified alginates were dissolved at 4 0C in Krebs-Ringer-Hepes (KRH) with an appropriate osmolarity to a solution with a viscosity of 4 cps. The viscosity of an alginate solution is determined by the concentration of alginate but different alginates have different viscosities. This implies for the intermediate-G solution a concentration of 3% and for the high-G a 2% solution to obtain a viscosity of 4 cps. Before application the solutions were sterilized by 0.2 µm filtration.

CHO-K1 cells were trypsinized, washed three times and brought in suspension with a defined volume of alginate to reach a concentration of $2x10^7$ cells per milliliter alginate.

For producing capsules, the alginate/CHO-K1 mixture or alginate without cells (i.e. for the *in vivo* experiments) were converted into droplets using an air-driven droplet generator as previously described [21]. The alginate droplets were transformed to alginate beads by gelling in a 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution for at least 5 min. Subsequently, the Ca-alginate beads were suspended for 1 min in Krebs-Ringer Hepes buffer containing 2.5 mmol/l $CaCl_2$. The diameters of the capsules were measured with a dissection microscope (Bausch and Lomb BVB-125, and 31-33-66) equipped with an ocular micrometer with an accuracy of 25 µm. The capsules had a diameter between 600 and 800 µm.

Animals and surgery

Female C57BL6(immunocompetent mice), age 6 to 8 weeks, were purchased from Harlan. Animal care was in accordance with institutional guidelines. Animal experiments were approved by the animal ethical committee of our institution.

Microcapsules were implanted in the cerebrum of C75BL6 mice as follows. C57BL6 mice were placed in a Kopf stereotactic frame (David Kopf Instruments, Tajunga, Canada) after intraperitoneal administration of a cocktail of anesthetics (Ketamine (75mg/kg)/Medetomedine (1 mg/kg)). Implantation was performed with the guided stereotactic implantation system. A burr hole was prepared, which was large enough for a needle with an inner diameter of 0.8 mm and an outer diameter of 1.2 mm. The alginate beads had a diameter of 700 µm. The coordinates for implantation of the beads were Bregma (x = 0) 2,5 mm to the left (y = -2,5) and 3 mm into the depth (z = 3). A microscope (Zeiss) was used to execute every step of the implantation. The skin was sutured with ethilon. Animals received postoperative an antidote (atipamezol-hydrochloride) and recovered on a heating plateau.

To assess the integrity of capsules before implantation, samples of capsules were meticulously inspected for the presence of irregularities or broken parts in the capsule membranes by using a dissection microscope.

To detect physical imperfections and to assess overgrowth after implantation, samples of adherent capsules recovered by excision were fixed in pre-cooled 2% paraformaldehyde, buffered with 0.05 M phosphate in saline (Ph 7.4), and processed for glycol methacrylate (GMA) embedding [22]. Sections were prepared at 2 µm, stained with Tonadine blue stain, and applied for determining the presence or absence of overgrowth.

Assays

Encapsulated CHO-K1 producer cells were double stained with acridine orange (AO) and propidium iodide (PI) (Sigma St. Louis, USA) to assess cell viability. AO staining was used to provide a green staining to detect living cells and PI to label dead cells with a red stain. AO and PI were dissolved in PBS to reach a final concentration of 6,7 µM for AO and 75 µM for PI. This solution was mixed on a 1:1 basis with growth medium and incubation with the capsules was done for 10 minutes at 23 °C. The capsules were washed twice with KRH and stored on ice. Fluorescence was measured with a fluorescence microscope (Leica) using red and green filters.

Encapsulated CHO-K1 producer cells (high-guluronic acid and intermediate guluronic acid content; 800-1000 µm) were tested for mitochondrial activity using the WST-1 assay (Boehringer Mannheim, Germany). Encapsulated CHO-K1 producer cells were seeded in a 96-wells plate (10 capsules/well). Subsequently, 100 µl of fresh medium was added together with 10 µl of WST-1. Incubation time with WST-1 was 45 minutes. Optical density (OD) was measured through an ELISA plate reader at a wavelength of 420-480 nm. A wavelength of 650 nm was used as reference. WST-1 assays were done on day 1,2,3,4,7,9,11,14 and 16 after encapsulation.

Encapsulated (radius 700 µm, high-guluronic acid) CHO-K1 producer cells (20 capsules/well) were placed in growth medium (200 µl) for a maximum of 14 days and were allowed to produce scFv425:sTRAIL. Supernatant of consecutive days was removed and applied in a viability assay to test the cytotoxic capacity of the released scFv425:sTRAIL. Cell death was assessed using a MTS assay according to standard procedures. Briefly, SW948 colon carcinoma cells, which are extremely sensitive to scFv:sTRAIL proteins, were seeded in flat bottom 96-well plates at a density of 3 x 10^4/well in 100 µl of growth medium. Cells were allowed to adhere overnight. Subsequently CHO-K1 producer supernatant of the consecutive days (150µl) was added. ScFv425:sTRAIL supernatant with a known concentration of the scFv:sTRAIL protein was used as a control. SW948 cells incubated with only growth medium were used as reference for both experimental groups. Incubation with the supernatant was done overnight followed by analysis of cell death with a MTS assay. Survival of cells was calculated using the formula; % cell death = ODexp /ODmedium x 100%.

A172 spheroids were individually placed in a 96 wells plate containing the appropriate growth medium. Spheroids were allowed to attach overnight to the wells. Thereafter the spheroids were exposed to 2, 5 or 30 alginate capsules containing the CHO-K1 (scFv425:sTRAIL) producer cells. Wells with a single spheroid without capsules added were used as control. Supernatant with scFv425:sTRAIL proteins collected from non-encapsulated producer cells was used as a control. EMD72000 (Merck), an anti-EGFR antibody, was used to block the toxic effect of the scFv425:sTRAIL protein. The

orthogonal diameter of each outgrowing spheroid was measured for up to 150 hours by light microscopy with a calibrated ocular. The circular area of cellular outgrowth from the spheroid is an index of the survival capacity of the cell and its ability to migrate.

Statistical analysis

Results are expressed as mean ± SEM. Statistical comparisons were made with the Mann Whitney U test. A p-value < 0.05 was considered statistically significant.

Results

Biocompatibility of intracranial implanted alginate capsules

Prior to implantation, we found the microcapsules to be perfectly spherical and without imperfections such as strains or broken membrane (Figure 1, pg 188).

Although intermediate-G alginate capsules are considered more fragile than high-G capsules, we found that with both types of alginate the integrity of the capsules remained intact during the time of implantation since we found no signs of broken membranes after retrieval of the capsules at 14 days post implant.

The capsules did not provoke any obvious immunological response in the cranial implantation site since we found no cell adhesion (Figure 2A and 2B, pg 189) on the surface of the capsules or signs of immunological activation in the tissues in the vicinity of the capsules. This absence of a response was observed with both intermediate-G and high-G alginate.

In the sham group, we sacrificed mice at day 1 after surgery to study the tissue damage and inflammation by the implantation surgery. Surprisingly, we found in spite of the presence of a relative large cavity no inflammatory response in the surrounding tissues and only some depositions of red blood cells (Figure 2C, pg 189). Fourteen days after the sham operation the cavity is reduced in size and infiltrating erythrocytes have disappeared (Figure 2D, pg 189).

Since both types of alginates are biocompatible in the cranial site, we applied both the intermediate-G and high-G alginates in the subsequent in vitro studies to investigate the compatibility with the enveloped cell-type.

Growth and viability of CHO-K1 producer cells after encapsulation

Figure 3 (pg 190) shows that the producer cells maintain their capacity to expand their population after encapsulation. Figure 3A (pg 190) shows cells 1 day after encapsulation. The cells are relative small and an intercellular space is present between individual cells. Figure 3C (pg 190) shows producer cells with a normal size and an intercellular space that is virtually completely filled with CHO-K1 producer cells. After 30 days, there is a large central area of dead cells surrounded by a thin peripheral rim of living cells (green emission). Up to 16 days this is a relatively small percentage of the total amount of encapsulated producer cells. After that period, the amount of dying cells gradually increases. This gradual increase in the number of dead cells was observed in capsules prepared of intermediate-G and high-G alginate. In spite of the strong growth of the

cell-populations, the capsules remained adequate and intact. After 30 days occasionally outgrowth of some producer cells was found (data not shown).

Mitochondrial activity of encapsulated CHO-K1 producer cells (Figure 4)

Producer cells should not only grow and remain viable in the capsules but they should also remain biologically active. Therefore, we studied the mitochondrial activity at several time points after encapsulation.

Although it was never statistically significant, we found an effect of the type of alginate on the mitochondrial activity of the producer cells. With high-G alginate, the producer cells reached an optimum of mitochondrial activity at day 9 is activity gradually decreased after day 9 with a second elevation at day 30 (Figure 4A and 4B). With intermediate-G, producer cells showed a peak level in mitochondrial activity at day 7 with a gradual decrease in the period thereafter (Figure 4C).

Biological activity of the released scFv425:sTRAIL protein

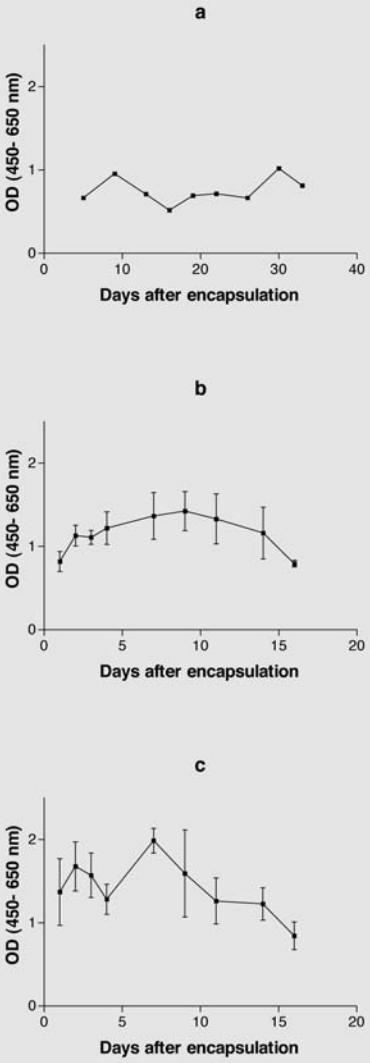
The biological activity of the released scFv425:sTRAIL protein was tested by assessing the apoptosis effect on colon carcinoma cells (SW948).

Supernatants from the encapsulated producer cells, collected one day after encapsulation, induced apoptosis in 10% of the cells (Figure 5). The apoptosis rate increased dramatically in the period thereafter. Supernatants starting from day 3 after encapsulation showed a raise in apoptosis induction with an optimum at day 8 (Figure 5). Apoptosis induction drops profoundly at day 14.

The apoptosis inducing effects of the scFv425:sTRAIL on the outgrowth of cells from A172 spheroids was studied (Figure 6). A stabilisation of outgrowth was established when 5 capsules with CHO-K1 scFv425:sTRAIL producer cells were added to the individual spheroid (Figure 6A). This stabilisation of outgrowth could be combined with induction of massive apoptosis when 30 capsules were administered. This effect was so profound that we could not reliably measure the orthogonal diameter of the spheroids.

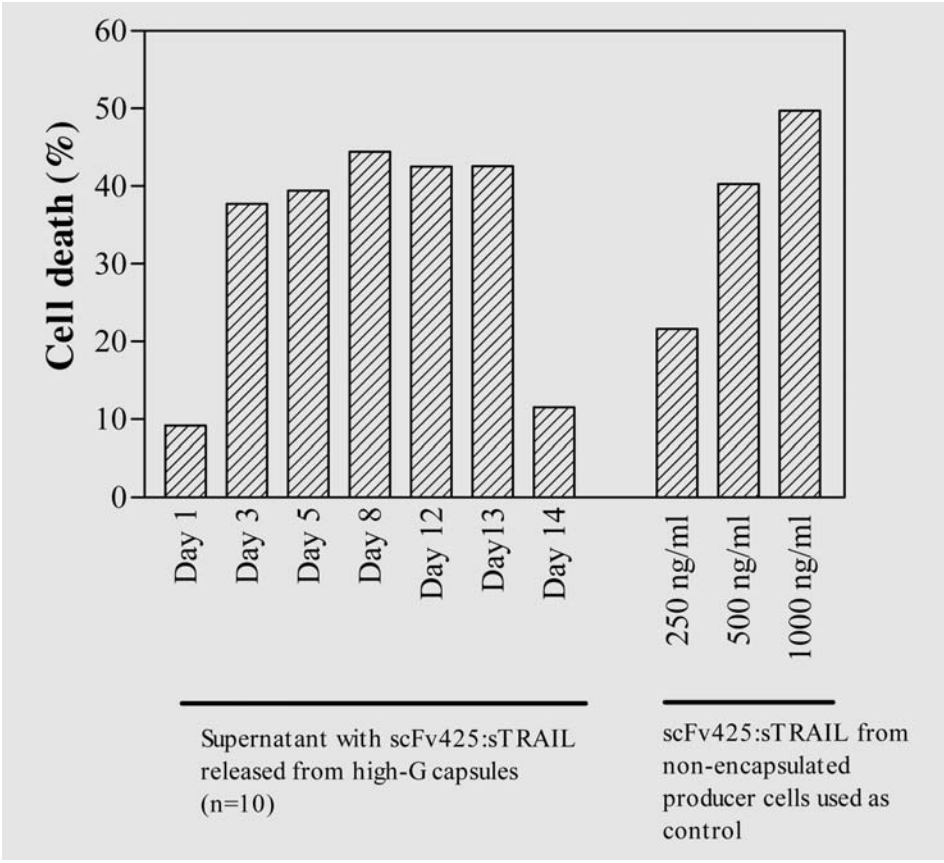
To confirm that this effect was due to the release of the scFv425:sTRAIL protein we blocked the effect of the protein by adding EMD72000, i.e. an anti-EGFR antibody. This did inhibit the death inducing effects of the 30 capsules on the spheroids (Figure 6B).

Figure 4



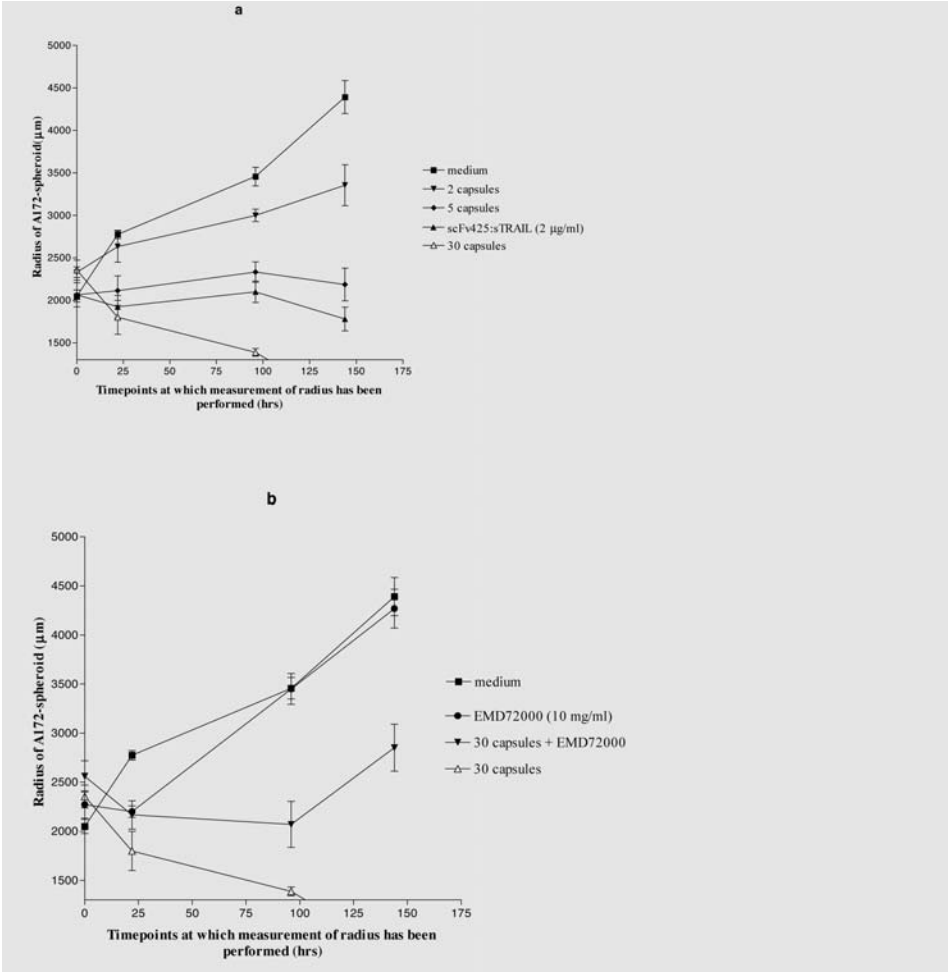
WST-1 assay measuring mitochondrial activity of encapsulated CHO-K1 producer cells (n=10). (a) The mitochondrial activity of the producer cells in high-G capsules (800µm) is at its peak at 9 and 30 days after encapsulation. (b) narrowing the period of interest between 0 and 15 days, again the peak is seen at 9 days for the high-G encapsulated producer cells.
(c) Mitochondrial activity of encapsulated producer cells in intermediate-G capsules (1000µm). peak activity is seen at day seven. WST-1 incubation is done for 45 minutes. OD was measured at an ELISA plate reader at 450 nm with a reference of 650 nm. Data represent means of 5 measurements, bars represent 95% CI.

Figure 5



Apoptosis inducing effect of scFv425:sTRAIL released from encapsulated CHO-K1 producer cells. SW948 cells (30.000cells/well) were seeded in a 96 wells plate. Cells were allowing overnight attachment to the base of the wells. Subsequently supernatant (150 µl), of consecutive days, containing the released protein from the capsules (n=10), was added to the various experimental wells. Supernatant containing scFv425:sTRAIL (150 µl) produced by non-encapsulated CHO-K1 cells was used as a positive control. Apoptosis in SW948 cells was set a 0%. Analysis was done with a MTS assay as described in Material and Methods.

Figure 6



(a) Inhibition of outgrowth due to scFv425:sTRAIL protein release from alginate capsules. A172 spheroids were individually placed in a 96 wells plate containing the appropriate growth medium. Spheroids were allowed to attach overnight to the base of the wells. Thereafter the spheroids were exposed to the alginate capsules containing the CHO-K1 (scFv425:sTRAIL) producer cells. The number of capsules added to the various experimental wells were 2, 5, 30 capsules. Medium containing wells with a spheroid without capsules were used as control. Supernatant with scFv425:sTRAIL proteins collected from non-encapsulated producer cells was used as a control. (b) EMD72000 an anti-EGFR antibody was used to block the scFv425-targeting moiety of the scFv425:sTRAIL protein. The orthogonal diameter of each outgrowing spheroid was measured for up to 150 hours. A light-microscope with a calibrated ocular was used for measurement. The circular area of cellular outgrowth from the spheroid is an index of survival and the ability of cells to migrate. Presented are means of four replicate wells, bars represent SEM.

Discussion

In the present study, we analyzed the applicability of encapsulated producer cells for chronic release of cytotoxic agents in the vicinity of tumor cells. First, we showed that CHO-K1 cells transfected with the gene encoding for the scFv425:sTRAIL protein can, after encapsulation, produce stable scFv425:sTRAIL proteins with preservation of its death inducing capacity. Secondly, biocompatibility of the alginate capsules in mice brains was found.

In the present study, we also demonstrated that producer cells could maintain normal growth capacity in capsules prepared of both intermediate and high-G alginate. However, after 16 days of culture some cell-death was observed in the capsules. This is usually caused by the fact that the cell number rapidly increased that competition occurred for the available nutrients [23]. After 30 days, this results in necrosis of cells in the center of the capsules since these cells have the lowest amounts of nutrients and oxygen to their disposal [16,23]. Occasionally we found some cells growing out of the capsules after prolonged periods of culture. This has been reported before but is usually explained by breakage of the alginate capsule [16,23]. This breakage has not been observed in this study. The capsules within this study were, during the study period, intact. The explanation of extracapsular growth of cells within the system used in this study must be attributed to protruding cells that expand their daughter cells through the alginate capsule without destroying it. Our studies show no principle difference in efficacy between intermediate and high-G capsules. Both capsule types showed optimal biocompatibility and functionality of enveloped cells. However, since high-G capsules are more stable, durable and less fragile, it is advisable to apply high-G capsules instead of intermediate-G capsules for in vivo studies.

The concept of the application of scFv:sTRAIL proteins is described elsewhere [11]. In brief, the scFv:sTRAIL protein consists of a targeting moiety composed of a single chain antibody fragment against the EGF receptor. The effector part of the scFv:sTRAIL protein is the death inducing ligand TRAIL. It is proven that targeting of TRAIL enhances its specificity and killing capacity [11]. To test this in this study, we competitively blocked the targeting moiety of the protein by adding EMD72000 (i.e. an EGF receptor-blocking agent) to the outgrowth experiment (Figure 6). We showed that EMD72000 successfully abrogated the inhibitory outgrowth effects of the scFv425:sTRAIL protein.

The lack of effect of the scFv425:sTRAIL protein in the first day after encapsulation (Figure 5) is plausibly due to lack of a sufficient concentration of scFv425:sTRAIL protein for exerting an effect since in control scFv425:TRAIL (2.07µg/ml) supernatants collected from non-encapsulated CHO-K1 producer cells we found a maximum of 50% cell death at a concentration of 1000 ng/ml (Figure 5). The drop in apoptosis activity at 14 days after encapsulation is concordant with the decrease in mitochondrial activity measured

by the WST-1 assay (Figure 4). The reason for this drop could be the relative shortage of nutrients within the center of the capsule. This lack of nutrients manifests itself with a lower metabolism of the producer cells and consequently less production of the scFv425:sTRAIL protein. However literature shows that the capacity of the producer cells to produce cytotoxic agents can hold on for periods far longer than 30 days [16,23]. It could be that CHO-K1 cells are not the ideal producer cells for encapsulation.

The second rise in mitochondrial activity (Figure 4) could well be the effect of the few cells outgrowing the capsule and thereby gaining more nutrients with higher mitochondrial activity as a result. We found that a higher number of capsules was required to achieve desired killing rates (Figure 6). To avoid multiple traumas at the cerebral implantation site it is advisable to apply a limited amount of capsules. Therefore, approaches should be designed to increase the production capacity. A conceivable approach is genetic manipulation to increase the number of protein copies in the cell and thereby creating a higher protein production. Another, option is to apply more smaller capsules since smaller capsules have a higher surface to volume ratio which is associated with better nutrition (i.e. higher cell-survival) and better kinetics of diffusion of the cytotoxic agents.

The next step in analyzing the feasibility of alginate encapsulated (scFv:sTRAIL protein) producer cells in treating brain tumors is to implant encapsulated producer cells in the brain of mice with and without an intracerebral tumor. Diffusion of the protein within the tumor, mass effect of the intracerebral lesion, edema surrounding the tumor, brain compliance and cerebro spinal fluid flow will be factors influencing the in vivo efficacy of the micro encapsulation method and must be analyzed before any prediction can be made if the micro encapsulation method will be a future therapy in the treatment of the intracerebral neoplasm.

Acknowledgements to: F Zuiderveen, R Horn and L van Genne for their technical assistance.

References

1. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H.: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104: 155-162, 1999
2. Chinnaiyan, A. M., Prasad, U., Shankar, S., Hamstra, D. A., Shanaiah, M., Chenevert, T. L., Ross, B. D., and Rehemtulla, A.: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 97: 1754-1759, 2000
3. Arizono, Y., Yoshikawa, H., Naganuma, H., Hamada, Y., Nakajima, Y., and Tasaka, K.: A mechanism of resistance to TRAIL/Apo2L-induced apoptosis of newly established glioma cell line and sensitisation to TRAIL by genotoxic agents. Br J Cancer 88: 298-306, 2003
4. Zhang, X. D., Nguyen, T., Thomas, W. D., Sanders, J. E., and Hersey, P.: Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. FEBS Lett 482: 193-199, 2000
5. Roth, W., Isenmann, S., Naumann, U., Kugler, S., Bahr, M., Dichgans, J., Ashkenazi, A., and Weller, M.: Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem Biophys Res Commun 265: 479-483, 1999
6. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H.: Tumoricidal activity of tumor necrosis factor-related apoptosis- inducing ligand in vivo. Nat Med 5: 157-163, 1999
7. French, L. E. and Tschopp, J.: The TRAIL to selective tumor death. Nat Med 5: 146-147, 1999
8. Kim, K., Fisher, M. J., Xu, S. Q., and El Deiry, W. S.: Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 6: 335-346, 2000
9. Wajant, H., Moosmayer, D., Wuest, T., Bartke, T., Gerlach, E., Schonherr, U., Peters, N., Scheurich, P., and Pfizenmaier, K.: Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 20: 4101-4106, 2001
10. Knight, M. J., Riffkin, C. D., Muscat, A. M., Ashley, D. M., and Hawkins, C. J.: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells.
11. Bremer, E., Kuijlen, J., Samplonius, D., Walczak, H., de Leij, L., and Helfrich, W.: Target cell-restricted and -enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. Int J Cancer 109: 281-290, 2004
12. Emerich, D. F., Winn, S. R., Hantraye, P. M., Peschanski, M., Chen, E. Y., Chu, Y., McDermott, P., Baetge, E. E., and Kordower, J. H.: Protective effect of encapsulated cells producing neurotrophic factor CNTF in a monkey model of Huntington's disease. Nature 386: 395-399, 1997
13. Mitsumoto, H., Ikeda, K., Klinkosz, B., Cedarbaum, J. M., Wong, V., and Lindsay, R. M.: Arrest of motor neuron disease in wobbler mice cotreated with CNTF and BDNF. Science 265: 1107-1110, 1994
14. Winn, S. R., Wahlberg, L., Tresco, P. A., and Aebischer, P.: An encapsulated dopamine-releasing polymer alleviates experimental parkinsonism in rats. Exp Neurol 105: 244-250, 1989
15. Read, T. A., Farhadi, M., Bjerkvig, R., Olsen, B. R., Rokstad, A. M., Huszthy, P. C., and Vajkoczy, P.: Intravital microscopy reveals novel antivascular and antitumor effects of endostatin delivered locally by alginate-encapsulated cells. Cancer Res 61: 6830-6837, 2001
16. Thorsen, F., Read, T. A., Lund-Johansen, M., Tysnes, B. B., and Bjerkvig, R.:

Alginate-encapsulated producer cells: a potential new approach for the treatment of malignant brain tumors. Cell Transplant 9: 773-783, 2000
17. Joki, T., Machluf, M., Atala, A., Zhu, J., Seyfried, N. T., Dunn, I. F., Abe, T., Carroll, R. S., and Black, P. M.: Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol 19: 35-39, 2001
18. de Vos, P., Hamel, A. F., and Tatarkiewicz, K.: Considerations for successful transplantation of encapsulated pancreatic islets. Diabetologia 45: 159-173, 2002
19. Uludag, H., de Vos, P., and Tresco, P. A.: Technology of mammalian cell encapsulation. Adv Drug Deliv Rev 42: 29-64, 2000
20. de Vos, P., van Hoogmoed, C. G., de Haan, B. J., and Busscher, H. J.: Tissue responses against immunoisolating alginate-PLL capsules in the immediate posttransplant period. J Biomed Mater Res 62: 430-437, 2002
21. Wolters, G. H., Fritschy, W. M., Gerrits, D., and van Schilfgaarde, R.: A versatile alginate droplet generator applicable for microencapsulation of pancreatic islets. J Appl Biomater 3: 281-286, 1991
22. Fritschy, W. M., Gerrits, P. O., Wolters, G. H., Pasma, A., and van Schilfgaarde, R.: Glycol methacrylate embedding of alginate-polylysine microencapsulated pancreatic islets. Biotech Histochem 70: 188-193, 1995
23. Rokstad, A. M., Holtan, S., Strand, B., Steinkjer, B., Ryan, L., Kulseng, B., Skjak-Braek, G., and Espevik, T.: Microencapsulation of cells producing therapeutic proteins: optimizing cell growth and secretion. Cell Transplant 11: 313-324, 2002

Chapter 6

Exploratory study on Convection Enhanced Delivery of an EGFR-selective TRAIL fusion protein in a xenografted brain tumor model

Summary

Objective: Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) is an apoptosis-inducing protein that shows promising tumor-selective activity in various animal tumor models. Previously, we demonstrated that the tumor-selective activity of TRAIL can be enhanced when it is genetically fused to a tumor-specific antibody fragment. This is exemplified by fusion protein scFv425:sTRAIL which comprises the EGFR-blocking antibody fragment scFv425 genetically fused to human soluble TRAIL (sTRAIL). Treatment with scFv425:sTRAIL potently induces apoptosis in EGFR-positive tumor cell lines. Moreover, potent *in vivo* systemic anticancer activity was previously demonstrated when scFv425:sTRAIL was adenovirally introduced in mice i.p. xenografted with EGFR-positive tumor cells. This prompted us to investigate the efficacy of convection enhanced delivery (CED) of scFv425:sTRAIL in the brain of mice that were stereotactically xenografted with EGFR-positive tumor cells.

Methods: The EGFR-restricted activity of scFv425:sTRAIL was assessed *in vitro* using a panel of human cancer cell lines, including SW948 (colorectal carcinoma), A431 (vulva carcinoma), SK-N-MC (neuroblastoma) and glioma cell lines U87, A172, U373, U251 and Hs683. This analysis was used to identify an EGFR-positive cell line that was particularly sensitive to scFv425:sTRAIL treatment. Subsequently, *in vivo* experiments were performed in the cerebrum of SCID mice that were stereotactically xenografted with the respective tumor cells. Convection enhanced delivery (CED) using an Alzet® osmotic pump was applied to intracerebrally, intratumorally infusion of scFv425:sTRAIL or a placebo. Pre- and post treatment MRI was used to assess tumor outgrowth and *in vivo* anti-tumor activity of the scFv425:sTRAIL fusion protein.

Results: *In vitro* analysis demonstrated differential sensitivities of the various human tumor cell lines towards scFv425:sTRAIL treatment. Cell lines SW948 and Hs683 proved to be particularly sensitive to scFv425:sTRAIL treatment. Since the tumor grafting of Hs683 cells proved to be poor, SW948 cells were selected to be stereotactically xenografted in the cerebrum of SCID mice. Pre-treatment MRI analysis confirmed the out-growth of SW948 cells in the brain. Post-treatment MRI indicated that under the experimental conditions used, convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps was insufficient to inhibit tumor growth.

Conclusion: Potent EGFR-restricted anti-tumor activity of scFv425:sTRAIL was demonstrated towards a series of EGFR-positive tumor cells. However, under the experimental conditions described in this manuscript, convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps appears to insufficient to inhibit tumor growth. In our current model loading of the osmotic pump with higher concentrations of scFv425:sTRAIL, using pumps with higher output capacity, as well as starting the treatment at lower initial tumor burden may be necessary to evaluate for anti-tumor efficacy of this novel approach.

Acknowledgements: We thank F. Zuiderveen, J Dokter-Fokkens, I. Platteel and D. Samplonius for excellent technical assistance. We thank A. Veltien of the Department of Diagnostic Radiology, University Hospital St Radboud, Nijmegen for his assistance with the MRI studies.

Sources of support:
KWF research grant for clinical residents 2000-2001
Foundation “De Drie Lichten” (grant number 99/31)

Introduction

Currently, patients with glioblastoma multiforme (GBM) have a very dismal prognosis. The overall survival time after conventional treatment (surgery, radiotherapy) varies between 9 to 12 months. Temozolomide, a new chemotherapeutic agent, was recently added to the standard treatment of GBM. However, in a large multicenter trial Temozolomide in combination with conventional treatment increased overall survival for not more than 2,6 months [1]. Therefore, development of new drugs is urgently warranted. In this respected targeted approach appear to be particularly promising.

In primary GBM's EGFR is often overexpressed or may be present in a mutated and is constitutive activated form (EGFRvIII)[2-5]. Various EGFR-targeted approaches, including antibodies and derivatives thereof, have been evaluated in *in vitro* and *in vivo* studies with promising results [6-11]. In particular, EGFR-selective agents that block oncogenic EGFR-signaling may have clinical importance since they render tumor cells more sensitive to therapeutic apoptosis induction.

In this respect a promising candidate appears to be the Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL). TRAIL is a tumor-selective pro-apoptotic effector molecule that is expressed on various immune cells including T- and natural killer (NK)-cells on which it is involved in the elimination of virus-infected and transformed cells. TRAIL interacts with an elaborate receptor system comprising two agonistic receptors, TRAIL-R1 and TRAIL-R2, two antagonistic receptors, TRAIL-R3 and TRAIL-R4, and the soluble receptor osteoprotegrin (OPG). The various TRAIL receptors are widely expressed on a variety of normal tissues and malignant cell types. Initially, TRAIL-R3 and –R4 were thought to act as decoy receptors, protecting normal and TRAIL-resistant tumor cells from apoptosis. However, recent reports show no correlation between TRAIL-sensitivity and expression of either TRAIL-R3 or TRAIL-R4. Consequently, the mechanism for the tumor-selective activity of TRAIL remains elusive.

Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the death-inducing signaling complex (DISC). The DISC includes the adaptor protein FADD and the initiator procaspase 8. Efficient DISC assembly results in concomitant activation of initiator- and effector caspases (e.g. caspases 3, 6, 7) and ultimately leads to apoptotic cell death.

TRAIL is expressed as a homotrimeric transmembrane protein (memTRAIL) of which the extracellular domain can be proteolytically cleaved off, yielding soluble TRAIL (sTRAIL). Various recombinant forms of sTRAIL have been generated that shown potent tumoricidal activity both *in vitro* and in various xenografted tumor mouse models [6, 7] with no or minimal activity towards normal cells [12, 13]. In a previous study we evaluated the expression of TRAIL-R1 and TRAIL-R2 in primary glioblastoma multiforme (GBM) tumor tissue

and demonstrated that the expression of these receptors is an independent prognostic factor for survival [14].

However, the efficacy of conventional recombinant sTRAIL preparation is potentially hampered by the ubiquitous expression of TRAIL-receptors on numerous normal cells and tissues. In addition, TRAIL-R1 and TRAIL-R2 have differential cross-linking requirements for apoptosis signaling, with TRAIL-R2 being less sensitive to sTRAIL than TRAIL-R1. As a result tumor cells that express TRAIL-R2 over TRAIL-R1 are less sensitive to treatment with sTRAIL. Previously, we have demonstrated that these limitations can be largely overcome by fusing a tumor-selective antibody fragment (scFv) to the N-terminus of human sTRAIL. Particularly promising in this respect is fusion protein scFv425:sTRAIL, which selectively targets sTRAIL to human EGFR-positive cancer cells. Previously, we have shown that scFv425:sTRAIL selectively binds to EGFR-positive cancer cells, whereupon EGFR-mitogenic signaling is rapidly inactivated and cancer cells are sensitized to apoptosis[7]. Moreover, potent in vivo systemic anticancer activity was previously demonstrated when scFv425:sTRAIL was adenovirally introduced in mice i.p. xenografted with EGFR-positive tumor cells.

Recently, Kawakami et al. studied safety and distribution kinetics of an IL-13-targeted cytotoxin that was infused intracranially by either a bolus injection or applied by convection-enhanced delivery using Alzet pumps in an athymic nude mouse model of GBM. This study demonstrated showed that CED is superior to bolus injections with respect to safety and obtainable drug levels in infused brain tumors [15].

This prompted us to investigate the efficacy of convection-enhanced delivery (CED) of scFv425:sTRAIL in the brain of mice that were stereotactically xenografted with EGFR-positive tumor cells.

Material and Methods

Cell lines and primary tumor tissue

All cell lines used were purchased from the ATCC. The following cell lines are EGFR-positive: SW948 cell line (colorectal carcinoma), the glioma cell lines U87 (p53wt), A172 (p53m) U373 (p53m), Hs683 (oligodendroglioma), A431 (vulva carcinoma), where as cell lines SK-N-MC (neuroblastoma) and the Jurkat (human ALL T-cell line) are EGFR-negative. The U251MG (p53m, EGFR+) line was a kind gift of D. Bigner, Duke University, North Carolina, USA. Tranfectant Jurkat-EGFRvIII cells were generated using retroviral particles encoding EGFRvIII. All cell lines were maintained in standard growth medium (DMEM for A172, U373, Hs683, U87, and U251 and RPMI-medium for SW948 and SK-N-MC) supplemented with 10% FCS (16 % for the SW948) and cultured at 37ºC in humidified 5% CO_2 atmosphere, unless indicated otherwise. Primary glioblastoma tissue was derived from the pathology department.

Construction and production of fusion protein scFv425:sTRAIL

The fusion protein scFv425:sTRAIL was constructed and produced essentially as described previously [7]. Briefly, in the first multiple cloning site of vector pEE14, the high affinity antibody fragment scFv425 (VH-(G4S)3-VL format) was directionally inserted
120 using the unique SfiI and NotI restriction enzyme sites. In the second MCS a PCR-truncated 593-bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in-frame using restriction enzymes XhoI and HindIII, yielding plasmid pEE14-scFv425:sTRAIL. Subsequently, plasmid pEE14-scFv425:sTRAIL was transfected into Chinese hamster ovary K1 cells using FuGENE 6 reagent (Roche Diagnostics) according to the manufacturer's instructions, after which transfectants were selected by the glutamine synthetase system as described [16]. Single cell sorting using the MoFlo high speed cell sorter (Cytomation, Fort Collins, CO) established clone 100F1, stably secreting 2,4 µg/ml scFv425:sTRAIL into the culture medium.

Preparation of RNA from tissue samples and cell lines

Snap-frozen sections (10´10µm) or cell suspensions were placed in a tube containing 300 µl lysisbuffer with 2.1 µl β-ME. For RNA-isolations the RNA RT-PCR Miniprep kit (Stratagene) was used. cDNA synthesis was performed using random primed hexamers. In a PCR-tube 10 µl RNA solution, 1 ml random primed hexamers (300 ng) and 1 µl 10 mM dNTP-mix were incubated at 65°C for 5 min. Thereafter, mix was put directly on ice. Subsequently, 4 µl 5´ First-Strand buffer, 2 µl 0,1 M DTT and 1 µl RNaseOUT (a recombinant Ribonuclease Inhibitor) (40 units/µl, Invitrogen) were added. The mix

was incubated at 25 °C for 10 min at 42 °C , after which 1 μl SuperscriptII (200 units, Invitrogen) was added. This reaction mixture was incubated for 50 min at 42 °C after which the reaction was stopped by incubation at 70 °C for 15 min. The cDNA and RNA samples were stored at minus 20°C until used.

RT-PCR expression of TRAIL R1, TRAIL-R2, EGFR and EGFRvIII

For RT-PCR detection of EGFR and EGFRvIII mRNAs a primer set was designed based on respective published sequences using Primer design 3 software. PCR with forward primer ATG CGA CCC TCC GGG ACG and reverse primer GAG ATC GCC ACT GAT GGA G yielded 1107bp PCR product for wild-type EGFR and a 305bp PCR product for EGFRvIII.

For the RT-PCR of TRAIL-R1 and TRAIL-R2, two primer sets were designed by using the relevant published sequences (NM-003842, NM-147187 and NM-003844) and Primer design 3 software. PCR for TRAIL-R1 was performed using the forward primer 5'-AGAGAGAAGTCCCTGCACCA-'3 and reverse primer 5'-GTCACTCCAGGGCGTACAAT-'3; for TRAIL-R2 (variants) forward primer5'-GAT-GGTCAAGGTCGGTGATT-'3 and reverse primer 5'-TACGGCTGCAACTGTGACTC-'3. Standard PCR primers for the household gene GAPDH were used as reference.

In short, 1 μl of each cDNA was amplified using PCR Master Mix (Amersham Biosciences) under the following PCR conditions; 5 min. 94 °C, 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 90 s, followed by 70°C for 7 min. The respective PCR-products were run on a 1% agarose gel containing Ethidium bromide and analyzed with the Geldoc 1000 (Biorad).

FACS analysis of TRAIL-R1/R2, EGFR expression and scFv425:sTRAIL binding

Cells from the different cell lines used were trypsinized (0.1% trypsine), harvested and resuspended in 1 ml fresh culture medium. Cells were counted using a Coulter counter and were divided over several polystyrene FACS tubes (5 x 10^5 cells/tube) to analyse the EGFR and TRAIL receptors. TRAIL receptor expression was analyzed with an anti-TRAIL receptor- FITC conjugated antibody (0,1mg/ml; Alexis Biochemicals). The monoclonal anti-TRAIL antibody 2E5 (1mg/ml; Alexis Biochemicals) was used to detect the TRAIL domain of the fusion protein. EGFR analysis was performed using the anti-EGFR anti-body MAb425 (stock solution 5mg/ml). The goat anti-mouse PE-conjugated antibody (GaM-PE; DakoCytomation) was used as secondary antibody. All incubations were performed on ice for at least 50 min and followed by two consecutive wash steps using fresh medium. FACS analysis was performed as follows: undiluted scFv425:sTRAIL

supernatant (250 µl) was added to the cell suspension (5 x 10^5 cells) and incubated for 60 min. Cells were washed (2x), the medium was decanted and the cells were resuspended in fresh medium. Anti-TRAIL MAb 2E5 was added and incubated for another 50 min followed by washing steps. Subsequently, GAM-PE was applied and incubated for 50 min and followed by 2 wash steps, thereafter FACS analysis was performed according to standard procedures and protocols.

Viability assay

Viability of cells was assessed using a crystal violet assay according to standard procedures. In short, tumor cells were seeded in flat bottom 96-well micro culture plates at a density of 3 x 10^4/well in 100 µl DMEM supplemented with 10% FCS. After overnight culturing, the spent medium was removed and replaced by 200 µl fresh medium supplemented with scFv425:sTRAIL. Control wells contained unconditioned medium. Plates were reincubated overnight followed by removal of the supernatant and staining with crystal violet. Plates were evaluated by measuring optical density (OD) at 575 nm on an ELISA plate reader. Four wells were measured in each experimental and control group. Viability of cells was calculated using the formula; % viability = OD_{exp}/OD_{medium}. Cell viability was represented as percentage of the medium control.

Immunohistochemistry

Five µm frozen sections from the mouse brains with intracerebral tumor tissue were fixated with acetone for 10 min. and washed with phosphate buffered saline solution (PBS, 0.01 M phosphate buffer, 0.15M NaCl). Sections were blocked for endogenous avidin and biotin activity using an avidin/biotin blocking kit (SP-2001 vector laboratories, inc. Burlingame, CA 94010). The sections were incubated for 60 min. with anti-CEA antibody (69186, clone parlam 4, ICN biochemical, inc, Aurora, Ohio) diluted 1:10, EGFR antibody (ab-30, Abcam limited, Cambridge) diluted 1:200, TRAIL-R1 antibody (DR4, C-20, Santa Cruz biotechnology, inc) diluted 1:100 and TRAIL-R2 antibody (DR5, PC392, Oncogene research products, Boston) diluted 1:100. All antibodies were diluted in 1%BSA/ PBS. The endogenous peroxidase activity was blocked by pre-treatment with 1% H_20_2 in PBS for 30 min. After washing with PBS, the sections were incubated for 30 min. with the secondary biotinylated rabbit anti-mouse(E0413, Dakocytomation, Denmark A/S, for CEA and EGFR) or rabbit anti-goat (6165-08, Soutern biotechnology associates, inc, Birmingham, USA, for TRAIL-R1/DR4) or goat anti-rabbit (4050-08, Soutern biotechnology associates, inc, Birmingham, USA, for TRAIL-R2/DR5). All secondary antibodies were diluted 1:300 in 1%BSA/PBS. Specific staining was amplified using streptavidin-biotin amplification kit (K0377, Dakocytomation, Denmark A/S) for 30 min. The sections were treated with DAB for 10 min. and counterstained with haematoxylin/eosin.

Animals

Female SCID mice (CB17 HanHsd SCID) were purchased from Harlan England. Mice were 6 to 8 weeks old and weighted around 16 to 20 grams. Mice were kept in flow units and were fed according to a standard regimen for immune-compromised mice. The *in vivo* experiments were approved by the local animal ethical committee of the UMCG.

Intracerebral tumours and Alzet® osmotic pump implantation

SW948 colorectal carcinoma tumor cells (100.000 cells/5 µl) were implanted with a SFE needle in the left striatum of the SCID mice. Cells were harvested one hour before implantation, trypsinized, counted and maintained in 37 °C medium before implantation. Cell viability was tested before and after the intracerebral implantation.

A Kopf stereotactic frame was used to implant the cells at the following coordinates: bregma (x=0), 2 mm to the left (y=2) and 3 mm into the depth (z=3). A microscope was used to execute every step of the implantation. A burr hole was made with a drill at the above mentioned coordinates. With the guided stereotactic implantation system the needle with the tumor cells was introduced to a depth of 3 mm and withdrawn to a depth of 2,5 mm thereby creating a space for the tumor cells preventing possible backflow along the needle tract. One µl per min was infused; thereafter a one min interval was scheduled also to prevent backflow of tumor cells. After this interval again 1µl tumor cells was infused and the protocol repeated itself until the total volume of 5 µ was infused (100.000 cells). Fourteen days after tumor cell implantation a pre-treatment MRI scan of the mouse cerebrum was made. The day after the MRI scan, an Alzet® osmotic pump (Model 2001; 200 µl volume, 1µl/hr infusion speed, Alza Corporation, Palo Alto, California) was implanted subcutaneously in the neck fold and the head canule was inserted, through the already present burr hole, intratumorally and fixated on the skull with histoacrylate glue. The pump was filled with fusion protein scFv425:sTRAIL (2,4 µg/ml) (n=3) or a placebo (n=2). At day 12 the pump was removed and at day 13 a post treatment MRI was made. Subsequently the mice were terminated under general anesthesia. The mice brains were snap frozen in isopentane and stored in liquid nitrogen.

Magnetic resonance imaging

Imaging was performed on a MRI system equipped with a 7.0-tesla magnet (Dept. of Diagnostic Radiology, University Hospital St Radboud, Nijmegen, The Netherlands). For MRI examination, mice were anesthetized with an isofluorane/air mixture and were maintained at 37°C inside the magnet by using a heated, thermostated circulating water bath. A single-slice sagittal spin-echo sequence was used to confirm proper animal positioning

and to prescribe subsequent imaging. The following acquisition parameters were used: T1 parameters: TR: 400ms, TE 6 ms, 256 x 256 matrix. Slice thickness: 0,5 mm. Slice separation 0,5 mm. Total slices 20. FOV: 35 x 35 mm. T2 parameters : TR: 2500 ms, TE 35 ms, 512 x 256 matrix. Slice thickness: 0,5 mm. Slice separation 0,5 mm. Total slices 20. FOV: 40 x 40 mm. The z-gradient first moment was zeroed to reduce the dominant source of motion artefact. T1 weighted images were also performed with a contrast enhancing agent (Gadolinium). T1 weighted imaging with contrast enhancement were made with Gadolinium (200 µl) through tail vein infusion. The images were analyzed with MRIcro software version 1.33 to calculate the tumor volume before and after treatment.

Results

mRNA expression of EGFR, EGFRvIII, and TRAIL-R1/R2 in various cell lines and primary GBM tissue

Figure 1 shows expression of EGFR mRNA in the colorectal carcinoma cell line SW948, in primary GBM tissue samples and in glioma cell lines U87, A172 and U251. Wild-type Jurkat cells did not express EGFR or EGFRvIII. EGFRvIII mRNA was not detected in any of the cell lines evaluated, except for EGFRvIII-transfected Jurkat cells which showed a marked expression of EGFRvIII.. One of the primary GBM tissues showed strong expression of EGFRvIII mRNA, whereas expression in the other sample was weak. TRAIL-R1 and the splice variants of TRAIL-R2 (TRICK 2a and 2b) were detected in all cell lines and in the primary GBM tissue.

Detection of the EGFR, TRAIL-R1 and TRAIL-R2

FACS analysis (Figure 2) showed strong EGFR membrane expression in all glioma cell lines evaluated. As expected both SW948 and A431 cells, used here as positive controls, showed prominent EGFR expression, whereas SK-N-MC cells, used here as a negative control, showed no EGFR expression. The level of EGFR expression of the different cell lines, indicated as mean fluorescence intensity (MFI) varied from essentially 0 (for SK-N-MC cells) to a maximum of 2727 (for A431 cells). The glioma cell lines MFI's for EGFR expression ranged from 233 (A172 cells) and 709 (U373 cells), whereas SW948 cells had a MFI of 449.

TRAIL-R1 and TRAIL-R2 expression was highest in the SW948 cells. Typically, under the conditions used TRAIL-R1 expression was barely detectable in the other cell lines.

Pro-apoptotic anti-tumor activity of scFv425:sTRAIL

Treatment with scFv425:sTRAIL fusion protein resulted in massive apoptosis induction in the SW948 and Hs683 cells (Figure 2). The A172 cells and the U87MG cells showed intermediate sensitivity to scFv425:sTRAIL. In contrast U373, U251 and SK-N-MC cells (EGFR-negative) proved to be resistant to treatment with up to 300 ng/ml scFv425:sTRAIL fusion protein. Modest apoptotic cell death was detectable in A431 cells.

EGFR-restricted binding activity of the scFv425:sTRAIL fusion protein

The scFv425:sTRAIL tumor binding activity is represented in Figure 3. Of the panel of cell lines evaluated A431 cells showed the highest binding capacity for scFv425:sTRAIL.

This is fully in line with the extremely high expression levels of EGFR described for this cell line. Compared to glioma cell lines SW948 cells show relatively low levels of the scFv425:sTRAIL binding. Of the glioma cell lines evaluated highest binding capacity is found for U373 and U251 cells. As expected EGFR-negative SK-N-MC cells do not show any appreciable binding of scFv425:sTRAIL.

There is a linear correlation (Spearman r: 0,9; p=0.0046) between the amount of EGFR expression and the binding capacity of the scFv425:sTRAIL fusion protein (Figure 3).
No correlation could be found between the amount of EGFR (Spearman r: -0,5; p=0,2; Figure 3C), TRAIL-R1 (Figure 3D) or TRAIL-R2 (Figure 3E) expression and the anti-tumor activity of the scFv425:sTRAIL.

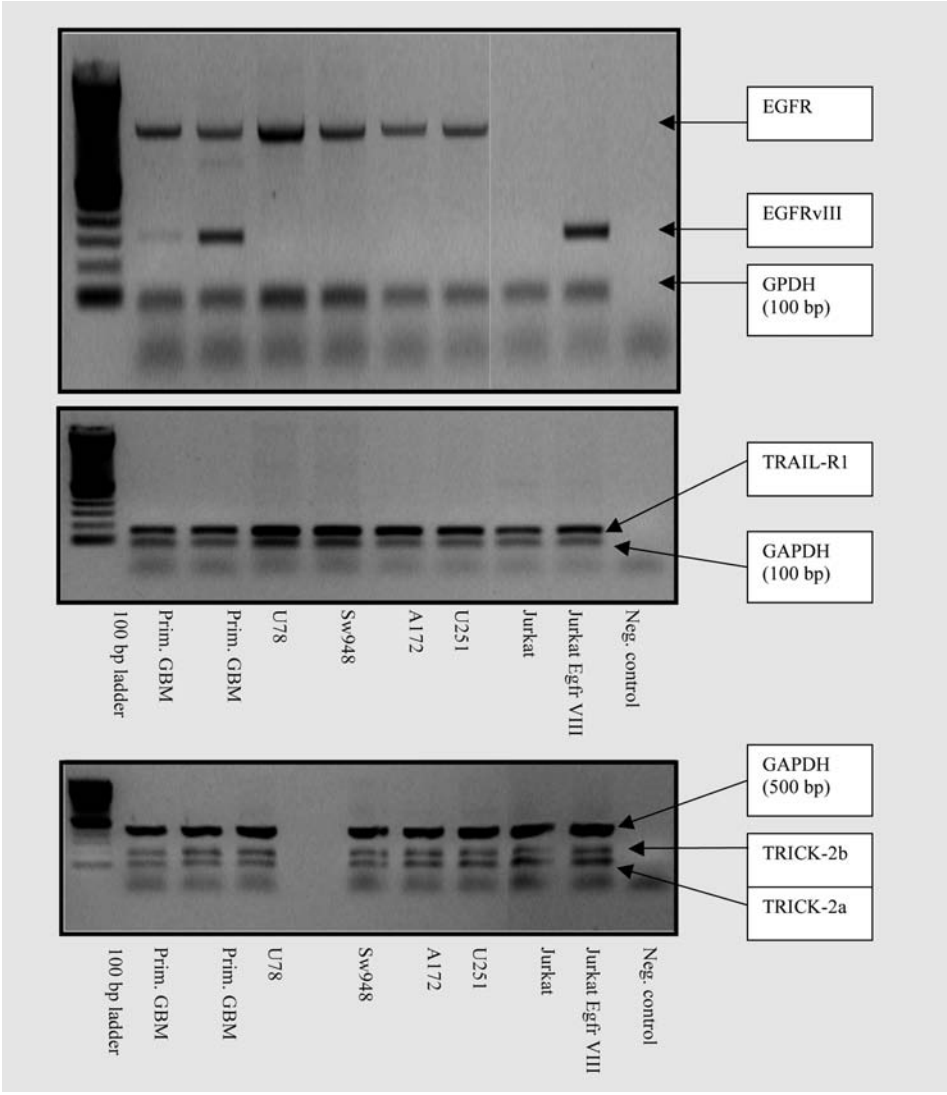
Efficacy of the scFv425:sTRAIL protein in a mouse brain tumor model

Table 1 represents the individual results of the tumor volume measurements of six mice with a SW948 intracerebral xenograft, before and after intracerebral treatment with a placebo or scFv425:sTRAIL fusion protein. T2 weighted images (WI) and T1WI (+ Gado) were used to measure tumor volumes. There is no significant difference between T2WI and T1WI (+Gado) in measurement of tumor volume. Mean tumor volume, before treatment, for the total group varied between 0,3 and 5,7 mm^3. There was no significant difference in pre-treatment tumor volume between the placebo and scFv425:sTRAIL group (Figure 4). After intracerebral infusional CED treatment with scFv425:sTRAIL there was a considerable increase in tumor volume for both the Placebo group (x8,4 and x12,9) and the scFv425:sTRAIL-treated group (x5,7; x10,3 and x12,5). No significant difference in tumor volume between both groups could be found (Figure 4). Mouse 4 had the smallest pre-treatment volume (0,3 mm^3) of the total group(n=6) however, even after scFv425:sTRAIL treatment, tumor progression could be visualized. Figure 5 presents the MR imaging of two representative mice brains. Pre-treatment scans of both groups show a contrast enhancing tumor (Figure 5A and 5C). After treatment, intracerebral progression of the tumor could be seen in both the placebo and scFv425:sTRAIL treated group (5 B and 5D).

Expression of EGFR and TRAIL receptors by the SW948 xenograft

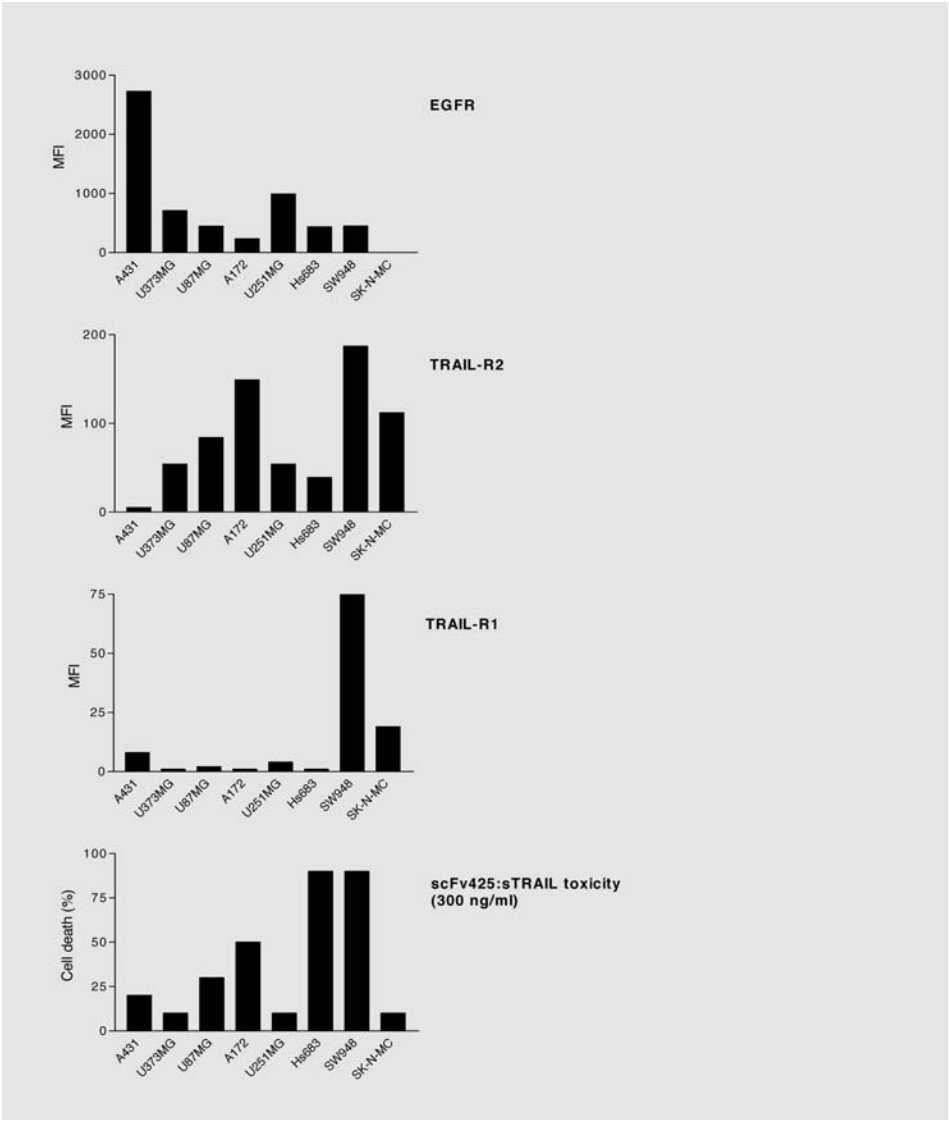
The intracerebral SW948 xenograft (Figure 6, pg 191) shows the typical morphological characteristics of a colorectal adenocarcinoma (overview) and highly expresses the colon embryonic antigen (CEA). After 30 days the intracerebral implanted SW948 cells still express the target antigen EGFR and TRAIL-R1 and TRAIL-R2.

Figure 1



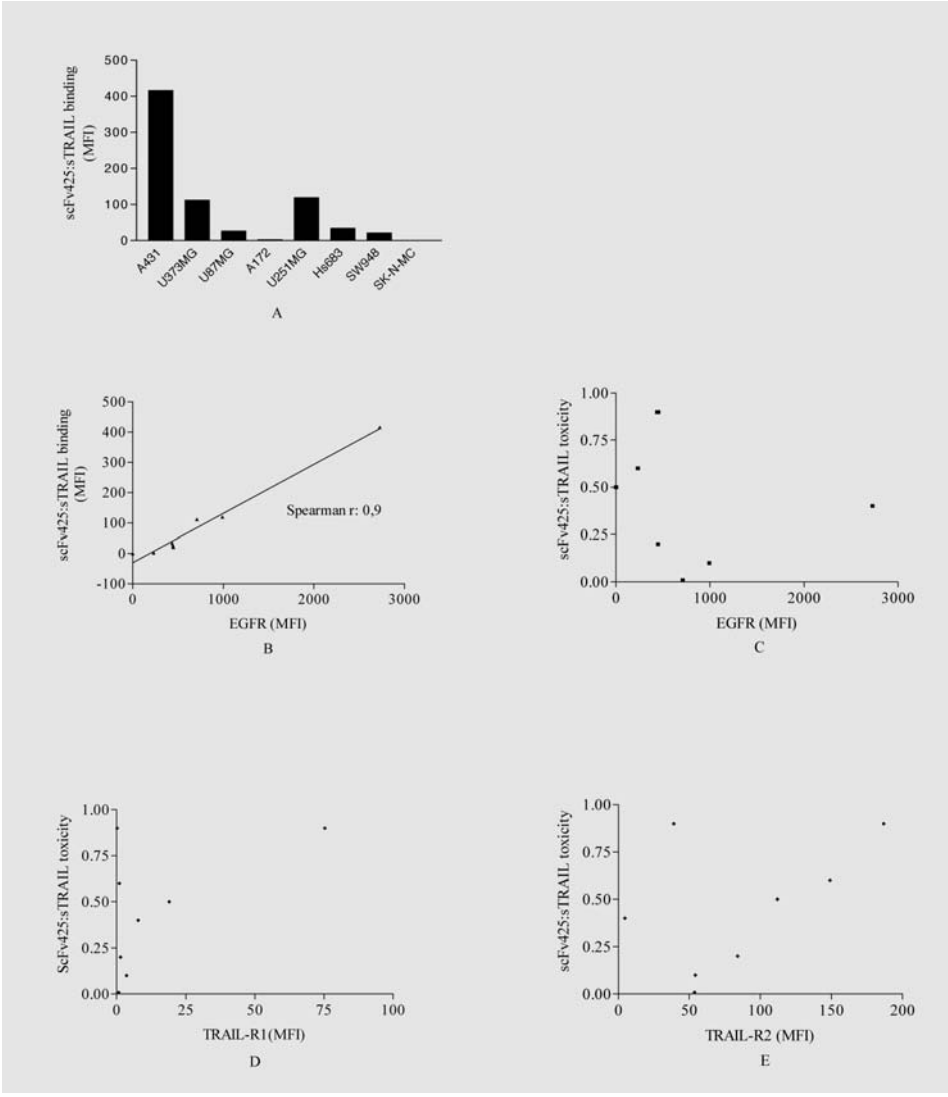
Representative samples of mRNA expression of the EGFR (1100 bp), EGFRvIII (310 bp) TRAIL-R1 (154 bp) and TRAIL-R2 splice variants (TRICK2a (93 bp) and TRICK-2b (181 bp)) in primary glioblastoma tissues and various human cell lines. All samples showed TRAIL-R1 and TRAIL-R2 variant mRNA expression. The negative control showed no expression. Jurkat and Jurkat-EGFRvIII were used as controls for the mutant EGFR. GAPDH (100 bp or 500 bp) was used as reference.

Figure 2



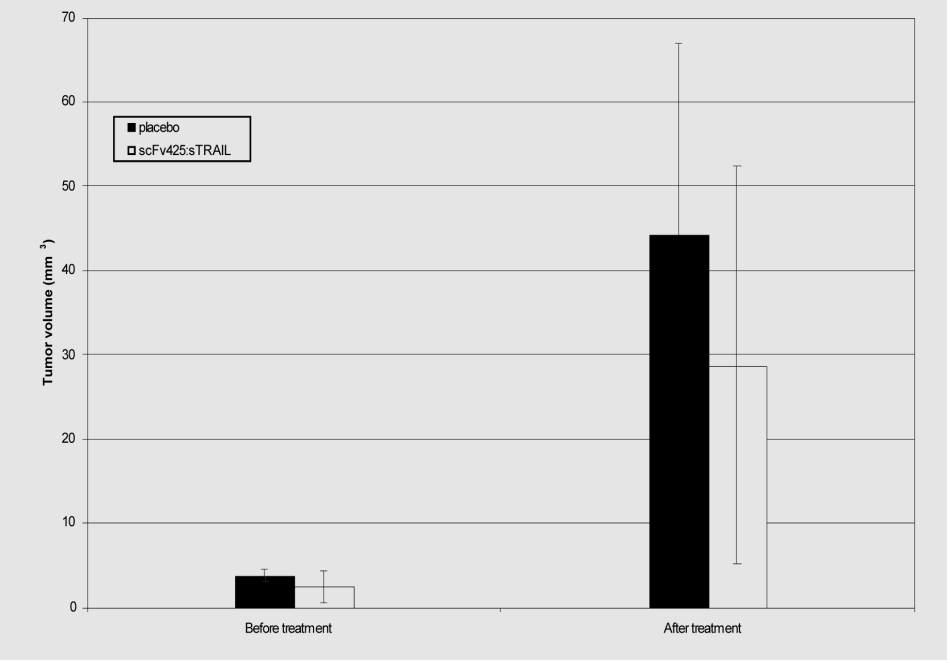
Receptor expression (EGFR, TRAIL-R2, TRAIL-R1) and scFv425:sTRAIL anti-tumor activity was analyzed in a panel of 8 cell lines. Cell death (%) by the scFv425:sTRAIL treatment (300 ng/ml) was analyzed with a Crystal Violet assay. The y-axis has different values to emphasize the large difference in receptor expression. A431 cells were used as positive control – and SK-N-MC cells were used as negative control for EGFR expression.

Figure 3



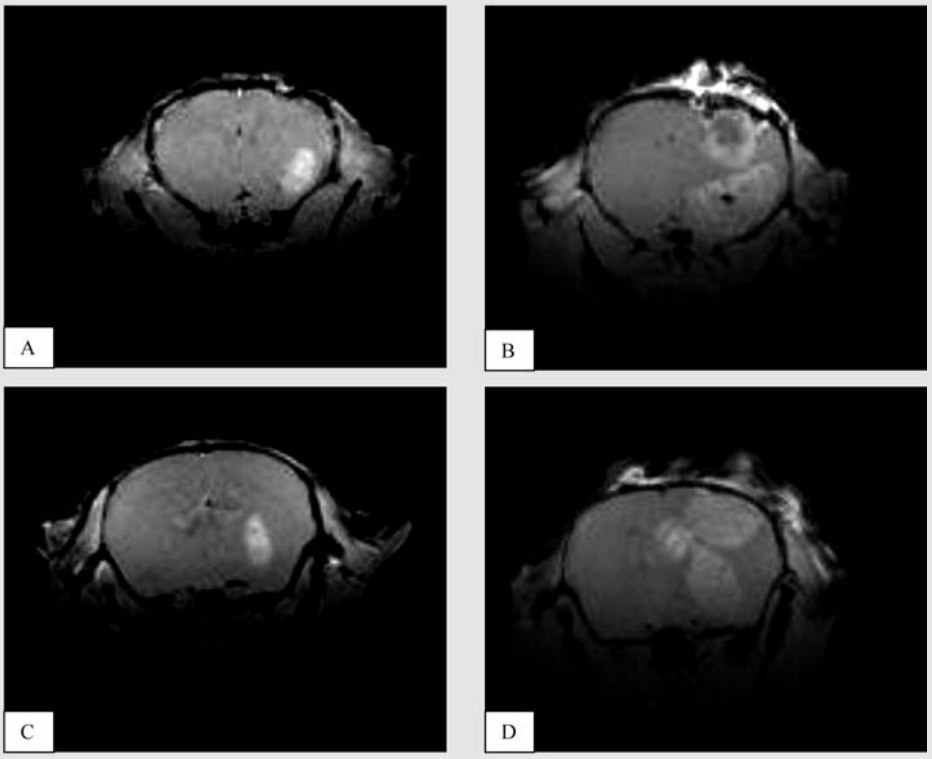
Binding of scFv425:sTRAIL to various cell lines (3A). Positive linear correlation between the scFv425:sTRAIL binding and EGFR expression (3B). The EGFR expression does not correlate with scFv425:sTRAIL toxicity (3C). No correlation was found between the TRAIL-R1 or TRAIL-R2 expression and scFv425:sTRAIL toxicity (3D and 3E).

Figure 4



Tumor volume evaluated by MR imaging (Gadolinium enhanced T1 WI) before and after experimental treatment. Bars represent SD.

Figure 5



Magnetic resonance imaging (Gadolinium enhanced T1 WI) of mice brain.
Figure 5A and 5B represent the same mouse brain before (A) and after (B) chronic intracerebral, intratumoral infusion with a placebo. Figure 5C and 5D represents the same mouse brain before (C) and after (D) intracerebral, intratumoral infusion of the scFv425:sTRAIL fusion protein. The placebo treated group consisted of 2 mice, the scFv425:sTRAIL group of 3 mice. An Alzet® osmotic pump with an infusion rate of 1 µl/hr was used to infuse the appropriate solution intratumorally for a period of 12 days.

Table 1

Table1: Intracerebral tumor volume analyzed by MR imaging, before and after intracerebral infusional treatment of a placebo (N=2) or scFv425:sTRAIL (N=3) fusion protein.

	Before treatment				After treatment		
	MR scan sequence	Voxel[1] number	Voxel dimension (mm^3)	Tumor volume (mm^3)	Voxel number	Voxel dimension (mm^3)	Tumor volume (mm^3)
Placebo	T2 WI	740	0,0064	4,736	NA[2]	NA	NA
	T1 WI +Gado	387	0,0098	3,7926	NA	NA	NA
Placebo	T2 WI	609	0,0064	3,8976	7399	0,0064	47,3536
	T1 WI +Gado	475	0,0098	4,655	6152	0,0098	60,2896
Placebo	T2 WI	537	0,0064	3,4368	4298	0,0064	27,5072
	T1 WI +Gado	343	0,0098	3,3614	2887	0,0098	28,2926
scFv425:sTRAIL	T2 WI	59	0,0064	0,3776	296	0,0064	1,8944
	T1 WI +Gado	38	0,0098	0,3724	218	0,0098	2,1364
scFv425:sTRAIL	T2 WI	895	0,0064	5,728	7675	0,0064	49,12
	T1 WI +Gado	383	0,0098	3,7534	4798	0,0098	47,0204
scFv425:sTRAIL	T2 WI	381	0,0064	2,4384	4429	0,0064	28,3456
	T1 WI +Gado	366	0,0098	3,5868	3791	0,0098	37,1518

1. Voxel: short for volume pixel, the smallest distinguishable box-shaped part of a three-dimensional image.
2. NA: not available, because mouse did not survive the implantation of the Alzet® pump.
The tumor volume is calculated by multiplying the voxel number with the voxel dimension.
Gado = gadolinium, a contrast enhancing drug.

Discussion

Novel GBM-selective approaches are urgently needed that have improved anti-tumor activity. A promising candidate for safe and cancer-restricted induction of apoptosis is the death inducing ligand TRAIL. TRAIL is a recently identified member of the TNF family of death ligands and shows selective apoptotic activity towards a variety of tumor cell types without toxicity for normal cells. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL) that can be proteolytically cleaved into soluble homotrimeric TRAIL (sTRAIL). Various forms of sTRAIL have been generated by recombinant DNA technology all showing potent *in vitro* and *in vivo* anti-tumor effects[13, 17-19]. TRAIL binds to an elaborate receptor system comprising at least two agonistic receptors, TRAIL-R1 and TRAIL-R2 and two antagonistic receptors, TRAIL-R3 and TRAIL-R4.

Indeed, TRAIL-R1 and TRAIL-R2 receptor expression are detectable in primary GBM tissue and represent independent prognostic factors for survival[14]. From our in *in vitro* results we conclude that comparable TRAIL-R1 mRNA and TRAIL-R2 mRNA levels are expressed in all the cell lines studied. However, FACS indicated that TRAIL-R2 cell surface expression is much more pronounced than TRAIL–R1. In fact, cell surface-expressed TRAIL-R1 is barely detectable. This is concordant with data from the literature[20]. From our RT-PCR and FACS results it can be concluded that TRAIL-R1 and TRAIL-R2 mRNA expression does not correspond with the cell membrane expression observed for these TRAIL receptors.

Here, we set out to pre-clinically evaluate our TRAIL-derivative scFv425:sTRAIL as a drug to eliminate malignant glioma cells.

Our *in vitro* analysis identified differential sensitivities of the various human cell lines evaluated towards scFv425:sTRAIL treatment. From this evaluation we conclude that the sensitivity of the various cell lines for scFv425:sTRAIL is not strictly dependent on the level TRAIL receptor expression at the cell surface. Differences and/or defects in intrinsic TRAIL-receptor signaling events may exist in the various cancer cell types. Indeed, it is reported that U373 glioma cells expresses very low levels of caspase 8 and FADD resulting in increased TRAIL-resistance [20].

We identified that SW948 colon cancer cells and Hs683 glioma cells are particularly sensitive to scFv425:sTRAIL treatment. Although the binding of the scFv425:sTRAIL fusion protein to the SW948 was relatively low (in comparison to some of the other cell lines), the sensitivity of SW948 cells to scFv425:sTRAIL proved to be extremely high. Since the xenograft acceptance of Hs683 cells was poor (data no shown), SW948 colon cancer cells were selected to be stereotactically xenografted in the cerebrum of SCID mice. Using this procedure over 90% of the injected mice developed SW948 xenografts within 14 days.

A major obstacle in treating patients with a brain tumor is delivering an anti-tumor drug to the site of the lesions. After systemic application of a cytotoxic drug, usually most of the drug is captured by the liver (first pass effect) or does not cross the blood brain barrier. Local application strategies, such as convection enhanced delivery (CED), that can bypass these factors are attractive and can strongly improve the efficacy of anti-tumor agents. In short, CED uses positive pressure infusion to achieve loco regional delivery of therapeutic agents through an intracerebral catheter, resulting in better intracerebral drug distribution than by diffusion alone [21, 22]. Therefore, we attempted to treat mice with an intracerebral xenografted human tumor by applying intracerebral CED of scFv425:sTRAIL.

Post-treatment MRI indicated that under the experimental conditions used CED of scFv425:sTRAIL by osmotic micro pumps was insufficient to inhibit tumor growth.

The failure of scFv425:sTRAIL to eliminate xenografted SW948 cells in the used model can be manifold. The local tumor microenvironment may counteract both the tumor penetration by scFv425:sTRAIL due to e.g. brain edema or enhanced intracerebral pressure. On the other hand, the tumor microenvironment may selectively inhibit the pro-apoptotic activity of scFv425:sTRAIL. This aspect is particularly difficult to study and was left unaddressed in the current study that was rather exploratory by nature than an in depth evaluation.

Next, we asked ourselves whether the biological activity of the scFv425:sTRAIL fusion protein was maintained in the reservoir of the Alzet® pump. Indeed, the anti-tumor activity of scFv425:sTRAIL was fully retained after 8 days of *in vitro* pumping at 37 °C (data not shown). Moreover, we evaluated if perhaps EGFR down-regulation in xenografted tumor cells may be responsible. Cryosections of the SW948 xenograft showed that both EGFR and TRAIL-R expression remained unaffected for a period of at least 30 days. Therefore, loss of either receptor type does not explain the observed failure.

The most obvious explanation for failure is the fact during treatment that not enough scFv425:sTRAIL is delivered at the site of the tumor. For our study we used an Alzet osmotic pump, Model 2001 with a 200 µl reservoir volume and an infusion speed of approximately 1µl/hr. The concentration of scFv425:sTRAIL used in the 200 µl reservoir of the pump was 2,4 µg/ml, adding up to a maximum of 0.48 µg scFv425:sTRAIL that becomes slowly available in the course of 12 days of therapy. At the starting point of treatment this minute amount of anti-tumor agent is clearly not sufficient to eliminate established SW948 tumors that already consist of several millions of tumor cells. Additionally, the proliferation rate of these cancer cells may easily outpace the elimination rate by the gradually infused scFv425:sTRAIL fusion protein. Moreover, dilutional effects of CSF, lymph drainage and tumor-induced interstitional fluid flow may further diminish the amount of scFv425:sTRAIL available to eliminate tumor cells.

In contrast, Nagane *et al* described an intracerebral mice tumor model (U87 glioma cells) in which mice were i.v. treated with FLAG-TRAIL at a concentration of 500 µg/day for 3 consecutive days and repeated for 3 weeks. This treatment regime lead to a prolonged survival of treated mice [23]. In the (somewhat arbitrary) assumption that only 10% of the total i.v. dose of infused FLAG-TRAIL will actually penetrate the brain, up to 50 µg FLAG-TRAIL may be available for eliminating tumor cells.

In our current model loading of the osmotic pump with higher concentrations of scFv425:sTRAIL, using pumps with higher output capacities, as well as starting the treatment procedure at lower initial tumor burdens may be necessary to evaluate for anti-tumor efficacy of our approach.

Since the time our study was performed alternative pre-clinical functional imaging modalities have become available in our institute that allow for a rapid and accurate evaluation of pre-treatment tumor load and *in vivo* follow up of the treatment effects in live animals. In particular Bio-optical Imaging based on bioluminescence, e.g. using the IVIS Imaging System (Caliper), has rapidly gained popularity. In this imaging approach tumor cells are first genetically modified to express luciferase, an enzyme derived from light-producing fireflies, before being xenografted into a mouse or rat. Upon injection with the substrate D-luciferin photons are emitted from the xenografted luciferase-modified tumor cells present in the living animal, which can be detected at the body surface of anesthetized animals using a sensitive CDD camera. The detected light emitted is digitally quantified and electronically displayed as a pseudocolor overlay onto a gray-scale animal image. Images and measurements of luminescent signals can be rapidly analyzed using dedicated imaging software and allow for a day-to-day evaluation of the *in vivo* growth and therapy-induced regression of tumors.

In conclusion, innovative treatment methodologies for glioblastoma multiforme are urgently needed. Animal models that better reflect the features of human GBM as well as novel per-clinical quantitative tomographic imaging technologies are currently under development. These innovations will allow for better ways of pre-clinical examining the effects of promising novel GBM therapeutics, including TRAIL and derivatives thereof.

References

1. Stupp R, Mason WP, van den Bent MJ, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987-996, 2005
2. Eppenberger U, Mueller H: Growth factor receptors and their ligands. J Neurooncol 22:249-254, 1994
3. Hoi SU, Espiritu OD, Kelley PY, et al: The role of the epidermal growth factor receptor in human gliomas: II. The control of glial process extension and the expression of glial fibrillary acidic protein. J Neurosurg 82:847-857, 1995
4. Hoi SU, Espiritu OD, Kelley PY, et al: The role of the epidermal growth factor receptor in human gliomas: I. The control of cell growth. J Neurosurg 82:841-846, 1995
5. Kuan CT, Wikstrand CJ, Archer G, et al: Increased binding affinity enhances targeting of glioma xenografts by EGFRvIII-specific scFv. Int J Cancer 88:962-969, 2000
6. Archer GE, Sampson JH, Lorimer IA, et al: Regional treatment of epidermal growth factor receptor vIII-expressing neoplastic meningitis with a single-chain immunotoxin, MR-1. Clin Cancer Res 5:2646-2652, 1999
7. Bremer E, Samplonius DF, van Genne L, et al: Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. J Biol Chem 280:10025-10033, 2005
8. Kuan CT, Wikstrand CJ, Bigner DD: EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer 8:83-96, 2001
9. Schmidt M, Vakalopoulou E, Schneider DW, et al: Construction and functional characterization of scFv(14E1)-. Br J Cancer 75:1575-1584, 1997
10. Shin DM, Donato NJ, Perez-Soler R, et al: Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. Clin Cancer Res 7:1204-1213, 2001
11. Werner MH, Humphrey PA, Bigner DD, et al: Growth effects of epidermal growth factor (EGF) and a monoclonal antibody against the EGF receptor on four glioma cell lines. Acta Neuropathol (Berl) 77:196-201, 1988
12. Ashkenazi A, Pai RC, Fong S, et al: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104:155-162, 1999
13. Roth W, Isenmann S, Naumann U, et al: Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem Biophys Res Commun 265:479-483, 1999
14. Kuijlen JM, Mooij JJ, Platteel I, et al: TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. J Neurooncol .78:161-171, 2006
15. Kawakami K, Kawakami M, Kioi M, et al: Distribution kinetics of targeted cytotoxin in glioma by bolus or convection-enhanced delivery in a murine model. J Neurosurg 101:1004-1011, 2004
16. Cockett MI, Bebbington CR, Yarranton GT: High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. Biotechnology (N Y) 8:662-667, 1990
17. Walczak H, Miller RE, Ariail K, et al: Tumoricidal activity of tumor necrosis factor-related apoptosis- inducing ligand in vivo. Nat Med 5:157-163, 1999
18. French LE, Tschopp J: The TRAIL to selective tumor death. Nat Med 5:146-147, 1999
19. Kim K, Fisher MJ, Xu SQ, et al: Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 6:335-346, 2000
20. Knight MJ, Riffkin CD, Muscat AM, et al: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. Oncogene 20:5789-5798, 2001

21. Laske DW, Ilercil O, Akbasak A, et al: Efficacy of direct intratumoral therapy with targeted protein toxins for solid human gliomas in nude mice. J Neurosurg 80:520-526, 1994
22. Laske DW, Morrison PF, Lieberman DM, et al: Chronic interstitial infusion of protein to primate brain: determination of drug distribution and clearance with single-photon emission computerized tomography imaging. J Neurosurg 87:586-594, 1997
23. Nagane M, Pan G, Weddle JJ, et al: Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res 60:847-853, 2000

Chapter 7

Effect of γ-radiation on rhTRAIL efficacy in glioblastoma multiforme cells

Jos M.A. Kuijlen, M.D.[1], Wilma. van Steenbergen, M.D.[1], Wilfred.F.A. den Dunnen, M.D., PhD.[2], Jan Jakob A. Mooij, M.D., PhD.[1], Harm H. Kampinga, PhD[3]

[1] Department of Neurosurgery
[2] Department of Pathology and Laboratory Medicine
[3]Department of Cell Biology, Section Radiation and Stress Cell Biology,
University Medical Center Groningen, The Netherlands

Submitted

Abstract

Objectives: It has been suggested that radiation can enhance the apoptosis-inducing efficacy of the TNF Related Apoptosis Inducing Ligand (TRAIL). Whether or not this is a general effect seen in all TRAIL receptor-positive cell lines and whether or not this also enhances the ultimate loss of clonogenicity of tumor cells remains to be elucidated. In this study we evaluated the effect of combined γ-radiation-TRAIL therapy in a glioblastoma cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints.

Methods: Glioblastoma A172 cells were treated by TRAIL, radiation or a combination of both treatments and cell death was analyzed by short term assays (Crystal Violet; FACS analysis) and a long term assay (clonogenic). Cell surface expression of the TRAIL receptor before and after radiation was evaluated by flow cytometry.

Results: Glioblastoma A172 cells expressed both the TRAIL R1 and –R2 receptor on the cell surface, but radiation did not increase the cell surface expression of these 2 receptors. Yet, the combination of radiation and TRAIL lead to a small synergy in apoptosis induction. However, this did not translate to synergy in ultimate loss of clonogenicity where radiation and TRAIL merely showed additive effects.

Conclusion: Up-regulation of TRAIL receptors by radiation does not seem a generic feature that is seen in all cell lines. Synergistic induction of apoptosis can occur, either via upregulation of TRAIL receptors (literature studies) or via dual triggering of synergizing signaling pathways that induce apoptosis (as suggestive from this study). This, however, does not (always) results in synergy at the level of clonogenic ability. Therefore when combining TRAIL treatment as an adjuvant to radiotherapy in the treatment of patients with malignant brain tumors, a synergistic interaction between the modalities is not to be expected.

Introduction

Glioblastoma multiforme tumors (GBM) tumors have been shown to be intrinsically resistant to radiation and although radiation has effect on overall survival, cures are not obtained. The development of new treatment modalities for patients with a GBM with the primary goal to enhance survival is an ongoing process. Such a new treatment modality could be the death inducing ligand TRAIL in combination with radiation. The subject of this paper is the death inducing effect of TRAIL in combination with radiation in a glioblastoma cell line.

Recombinant human TNF Related Apoptosis Inducing Ligand (rhTRAIL), a soluble death inducing ligand, induces cell death via binding to the transmembranous TRAIL-R1 and/or TRAIL-R2 receptors which triggers apoptosis. In a previous study we detected the presence of TRAIL-R1 and TRAIL-R2 receptors on primary GBM tumor tissue and showed the association of these receptors with tumor cell survival [1]. Various in vitro and animal in vivo experiments have shown the death inducing potential of TRAIL, without obvious toxicity for normal cells [2,3]. These results may implicate that rhTRAIL can be used as a therapeutic modality in the treatment of patients with various tumors, including those patients with a GBM. Unfortunately some GBM cell lines show also resistance to TRAIL [4,5].

An option may be to combine radiation and rhTRAIL to overcome resistance to either therapy. In several in vitro and ex vivo studies on non-GBM cells this was shown to be an effective combination therapy [6-11]. In fact, it has been suggested for leukemic and breast cancer cells that radiation upregulates TRAIL-receptors and enhances its cell surface expression, hereby enhancing the cellular sensitivity to TRAIL-mediated cell killing [10,11,8]. In contrast to this idea, Ciusani et al. [12] found no detectable effect of radiation (2Gy) on TRAIL receptor expression in two brain tumor cell lines (U373 and the SW1783). They also reported (data not shown) that apoptosis measurement (Annexin-V) after combinational treatment showed a modest (SW1783) to no (U373) increase in the number of apoptotic cells.

Most papers, addressing the combinational effect of radiation and TRAIL agonists, evaluate apoptosis as a short term end-point. Therefore, these studies do not take into account other modes of cell death that influence ultimate loss of clonogenicity of tumor cells. In other words, the same cells that would normally die late after radiation due to mitotic catastrophe may now die earlier from rhTRAIL induced (early) apoptosis due to radiation-induced upregulation of the TRAIL receptors, meaning that tumor cell death would be accelerated but ultimately not increased. This could lead to false conclusions regarding the potential enhancing effect of radiation on TRAIL and vice versa. In this study we therefore re-evaluated the effect of γ-radiation in combination with TRAIL therapy in a glioma cell line, evaluating both early apoptotic cell death and clonogenic ability as endpoints.

Materials and Methods

Cell lines

The glioblastoma cell line A172 was purchased from ATCC (Manassas, USA). A172 cells were cultured in DMEM, supplemented with 10% fetal calf serum (FCS) and 4 mM L-glutamine (Cambrex Bio Science) at 37°C in humidified 5% CO2 atmosphere.

TRAIL, antibodies and DiOC6

Recombinant human soluble TRAIL (rhTRAIL) was kindly provided by S. de Jong (University Medical Center Groningen, University of Groningen, Department of Internal Medicine, section Medical Oncology). Monoclonal (human) antibodies against TRAIL receptor TRAIL-R1 and TRAIL-R2 were purchased from Alexis Biochemicals (Benelux). Phycoerythrin-labeled goat anti-mouse IgG (GAM-PE) was used as a secondary antibody (DakoCytomation, Denmark). The cell-permeant green-fluorescent lipophylic dye DiOC6 was purchased from Molecular Probes, Eugene, OR.

Radiation

Cells were washed twice with phosphate buffered saline (PBS) and trypsinized, followed by neutralisation of trypsin with DMEM medium. After centrifugation (1500 rpm for 8 minutes) cells were resuspended in fresh medium at a concentration of 5 * 105 cells/ml (clonogenic assay) or 1 * 106 cells/ml (crystal violet assay, flow cytometry). In all experiments (unless mentioned otherwise), radiation was performed in suspension, using a 137-Cs gamma ray unit (IBL 637, CIS Biointernational, GIF sur Yvette, France) at a dose rate of 0,7895 Gy/min.

Flow cytometry

Flow cytometric analysis was performed at various intervals after radiation (8, 24 and 48 hrs). Cells were irradiated while plated (and attached) in T75 flasks. After radiation with 2 or 5 Gy, flasks were put back in a culture stove (37°C, 5% CO2). At the day of analysis, cells were washed twice with PBS and trypsinized. Cells were resuspended in fresh medium at a concentration of 5 * 105 cells/ml. After centrifugation (1600 rpm for 5 minutes) cells were resuspended in medium containing the various antibodies (aTRAIL-R1, aTRAIL-R2). Cells were incubated for 40 minutes on ice. After washing the cells with fresh medium, cells were incubated with the secondary antibody, GAM-PE, for 40 minutes (on ice). Cells were then washed twice with fresh medium and FACS analysis was performed using the ELITE flow cytometer.

Apoptosis was also measured using flow cytometry. Cells were harvested and radiated with 2 or 5 Gy. After radiation, cells were plated in 12-well plates (0,5 * 106 cells/well). Eight hours after radiation, rhTRAIL or medium was added to the different wells. The next day, all cells (adherent and non-adherent) were harvested. After centrifugation, cells were resuspended and DiOC6 was added. After incubation for 30 minutes at 37°C, cells were washed and analyzed by the Calibur flow cytometer.

Crystal violet viability assay

Tumor cell viability was assessed by a crystal violet assay. Cells were washed twice with PBS and trypsinized, followed by neutralisation of the trypsin with DMEM medium. After centrifugation (1500 rpm for 8 minutes) cells were resuspended in fresh medium and radiated. After radiation, cells were seeded in flat-bottom 96-well microculture plates at a concentration of 3*104 cells/well in 200 µl medium. Eight hours after radiation, 100 µl medium was removed and replaced by 100 µl fresh medium containing the various concentrations of rhTRAIL. Cells were reincubated overnight cells followed by a washing with PBS, staining with crystal violet solution and solubilized in 1% SDS. Absorbance was read at 575 nm by an ELISA-plate reader.

Clonogenic assay

Cells were washed twice with PBS and trypsinized. Cells were radiated and plated in 60 mm petri-dishes. Appropriate dilutions were made to yield 50 – 100 colonies per petri-dish. After plating the sample cells, 105 feeder cells (cells lethally radiated with 100 Gy) were added to each plate. Eight hours after radiation, rhTRAIL was added. Cells were stored in an incubator at 37°C and 5% CO2, for 12 – 14 days. After this period the medium was removed and cells were washed once with PBS. After fixating with 70% ethanol, cells were stained with 1% crystal violet solution and washed twice in tap water.
Colonies containing more than 50 cells were counted. Survival was calculated with the formula: (Number of colonies counted/number of cells plated) x (1/plating efficiency)
The plating efficiency (PE) is the colony count resulting from 100 untreated cells. In this study, the PE of A172 cells was 65%.

Data analysis and statistics

Interactions between TRAIL and radiation were analyzed by the fractional inhibition method13. When expressed as the fractional inhibition cell viability, additive inhibition produced by both inhibitors (Ia,b) occurs when Ia,b =Ia + Ib; synergism when Ia,b >Ia + Ib and antagonism when Ia,b< Ia + Ib. Differences between two groups were tested for

significance using a two-tailed non-parametric Mann-Whitey test. Differences between multiple groups (>2) were tested using one-way analysis of variance (ANOVA).

Results

Early cell death induced by rhTRAIL and radiation

First, we tested if combined treatment of radiation and recombinant human (rh) TRAIL would lead to enhancement of cell death in glioblastoma A172 cells using the crystal violet assay (Figure 1A). Rapid cell death after radiation alone was only 4%. Recombinant human (rh)TRAIL resulted in respectively 45% (10 ng/ml) and 65% (100 ng/ml) apoptosis. The combined treatment of radiation and rhTRAIL induced a significantly higher fraction of cell death than rhTRAIL alone (55% respectively 77% for 10 ng/ml and 100 ng/ml ($p=0.01$)). In Figure 1B it can be seen that the extent of rapid cell death after the combined treatment was significantly synergistic, albeit that the magnitude of the synergistic effect was small (rhTRAIL 10 ng/ml: $p = 0,04$; rhTRAIL 100 ng/ml: $p = 0,02$).

To test whether the rapid cell death was indeed due to increased apoptosis induction, we used flow cytometry. Figure 2 (pg 192) shows the FACS results of A172 cells after treatment with rhTRAIL, radiation or a combination of rhTRAIL and radiation. The fraction of apoptotic cells (cell under the pre G1 peak) increased from 5% (no treatment) to 40% and 77% after 10 or 100 ng/ml rhTRAIL respectively (Figure 2A, pg 192). After irradiation (2Gy) alone 11% of cells are apoptotic by these criteria (Figure 2B, pg 192). The combined treatment of radiation and rhTRAIL (10 ng/ml and 100 ng/ml) resulted respectively in 54.93% and 90.91% apoptosis. So, these data are consistent with the crystal violet data (Figure 1) and with the idea that this assay predominantly registers apoptotic cell death.

Clonogenic assay shows additive killing after combination treatment

To test whether the combined treatments not only accelerate cell death but also increase the ultimate level of cell death, we evaluated the ability of cells to form colonies after the single and combined treatments (Figure 3). Remarkably, whereas the early cell death assays only revealed 4% (crystal violet) respectively 11 % (FACS) cell death after 2Gy alone, only 25% of the cells were able to form colonies after this dose of radiation. This indicates that early apoptotic cell death only plays a minor role in radiation-induced cell death of A172 cells.
Cell survival was reduced in the non-irradiated group to 84,3%, 23,0% and 0,009% after 1, 5 and 10 ng/ml rhTRAIL respectively (Figure 3). Compared to the 40-45% apoptotic cell death after 10 ng/ml rhTRAIL in the short term assays (Figure 1 and 2 (pg 192)) this means that even TRAIL induces additional (delayed) cell death through non-classical pathways

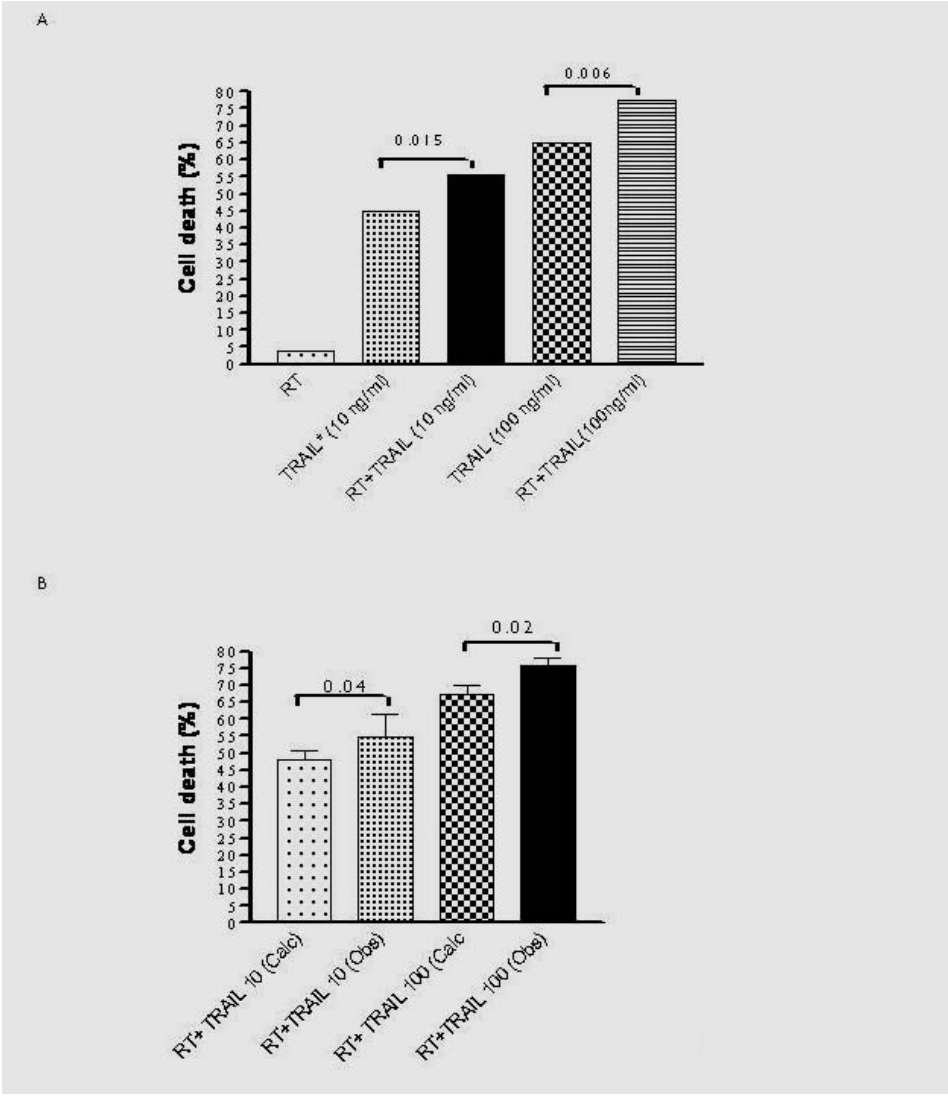
besides via rapid apoptosis alone. Treatment with 100 ng/ml rhTRAIL could not be analyzed due to the massive toxicity in the clonogenic assay (no colonies were detected).

As shown in Figure 3, the combined treatment with 2 Gy radiation and variable rhTRAIL concentrations (1, 5, 10 ng/ml) had merely additive effects on the clonogenicity of glioma cells. After treatment with radiation and 10 ng/ml TRAIL, there were no colonies found in plates where 40.000 cells were plated. Therefore, cell survival after this combined treatment was indicated as less than 0,0025% (shown by the arrow in Figure 3). So, although the combined treatments led to slightly more than additive rapid apoptosis, no enhancement of ultimate cell death was seen after the combination treatment.

TRAIL receptor expression is not increased after radiation

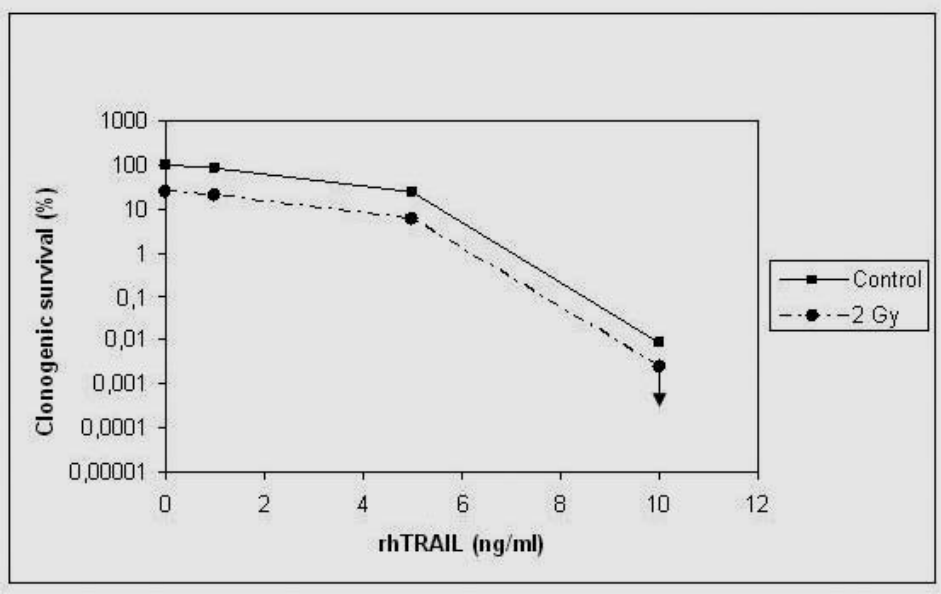
To test the idea that radiation may up-regulate TRAIL receptors at the cell surface and as such increase rapid apoptosis, we evaluated the cell surface expression of the TRAIL receptors TRAIL-R1 and TRAIL–R2 in A172 cells, by flow cytometry. Only 2,1% of the A172 cells expressed TRAIL-R1, whereas TRAIL-R2 was present on 99,2% of the cells (data not shown). After radiation (2 or 5 Gy), no significant radiation dependent changes were detectable in the expression of these two TRAIL receptors (Figure 4 and 5). The overall expression of TRAIL-R1 receptors did increase with time within all experimental groups, however, no statistical difference could be found in this increase between the control group and the radiation groups (Figure 4A). Also the number of TRAIL-R1 receptor positive cells remains extremely low over time and there was no significant increase after radiation (Figure 4B). Although upregulation of TRAIL-R2 receptor expression was seen after radiation, this effect was not significant and independent of time after radiation (Figure 5A). Also, the percentage of TRAIL-R2 positive cells was high and remained high with time without being affected by radiation (Figure 5B).

Figure 1



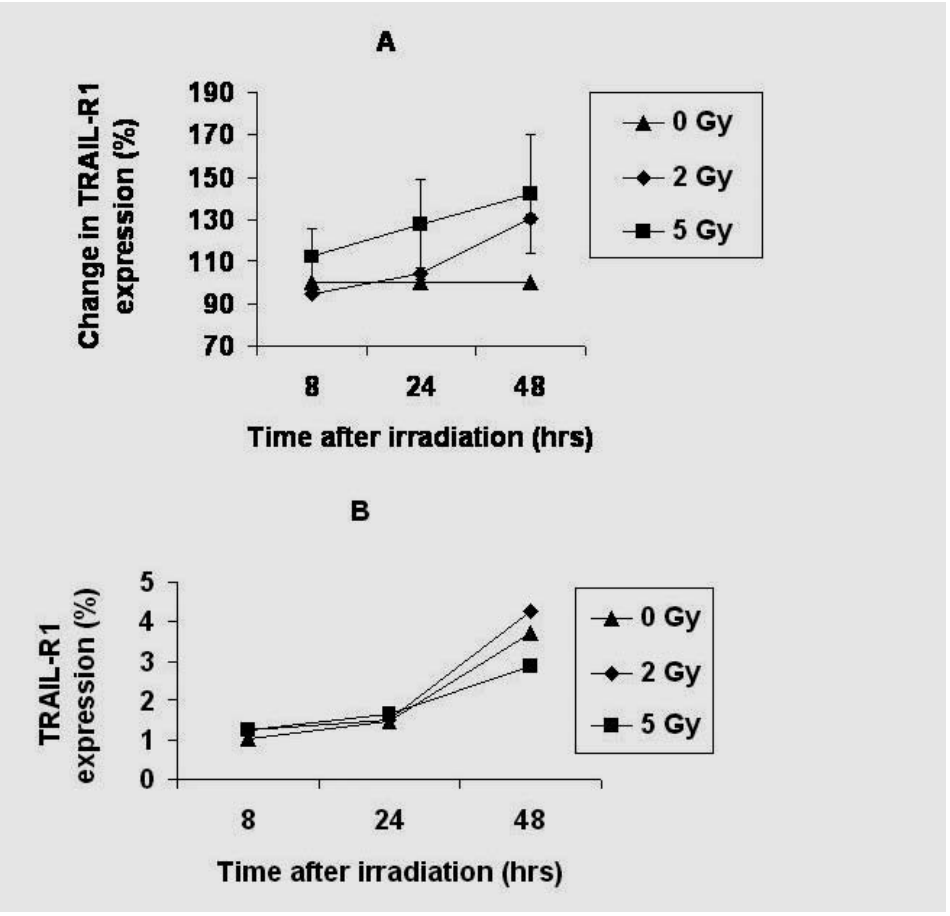
Rapid cell death induced as determined by the Crystal Violet assay after radiation, rhTRAIL (10 and 100 ng/ml) or a sequential treatment of radiation and rhTRAIL.
Panel A: Fraction of cell death after the respective treatments (= rhTRAIL).*
Panel B: comparison between the "observed" cell death and the calculated "additive" effect of both treatments (TRAIL 10= TRAIL 10 ng/ml). Data points represent the means and 95% confidence interval of 3 independent Crystal Violet assays

Figure 3



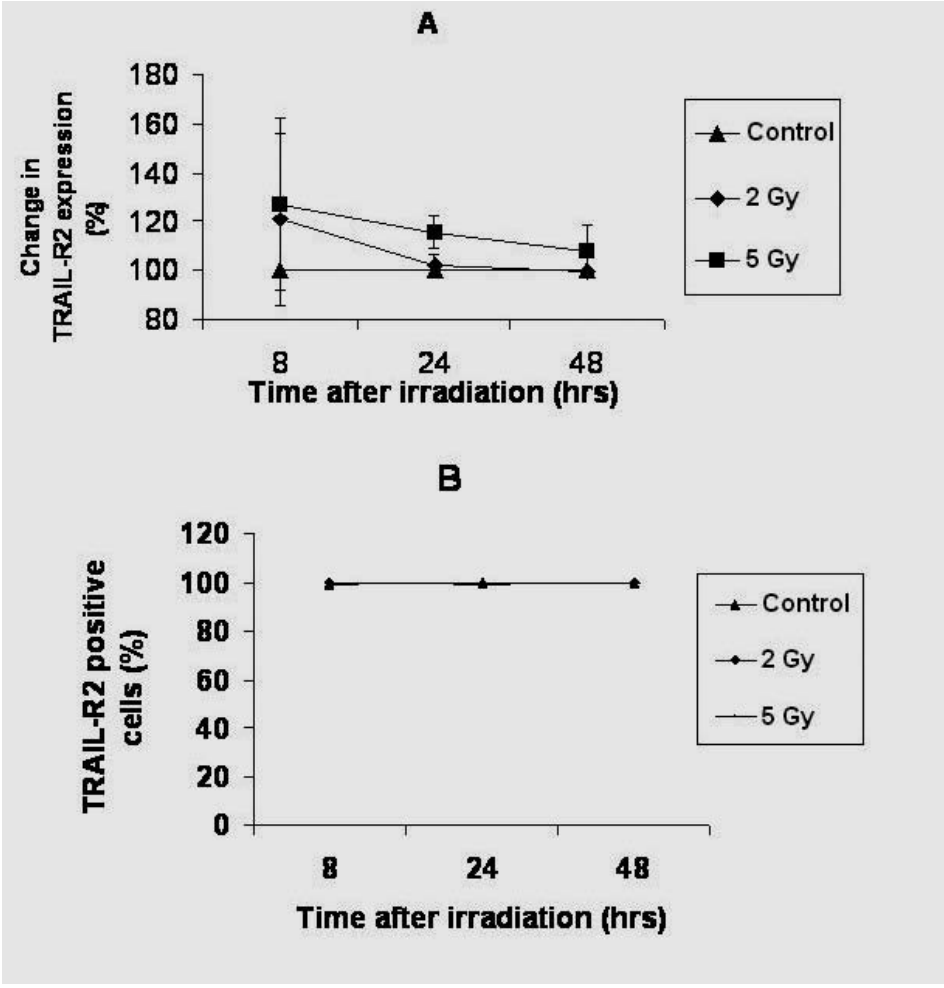
Clonogenic survival of A172 cells after treatment with graded concentrations of rhTRAIL either given alone (control, squares) or in combination with 2Gy irradiation (circles). The parallel lines indicate an additive effect of the combined treatment with radiation and rhTRAIL. The plating efficiency of the untreated A172 cells was 65 and set as 100%. ↓: Less than 0,0025% survival. This figure is a representative of 2 independent experiments performed in triplicate

Figure 4



Effect of radiation on TRAIL-R1 cell surface expression (panel A) and the percentage of TRAIL-R1 positive cells as measured by FACS analyses.
Panel A shows the mean fluorescence intensity as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares). The mean fluorescent intensity immediately after (sham) treatment of control cells was set as 100%. Bars represent SD of two independent experiments.
Panel B shows the fraction of cells with cell surface expression of the TRAIL-R1 receptor as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares).

Figure 5



Effect of radiation on TRAIL-R2 cell surface expression (panel A) and the percentage of TRAIL-R2 positive cells as measured by FACS analyses.
Panel A shows the mean fluorescence intensity as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares). The mean fluorescent intensity immediately after (sham) treatment of control cells was set as 100%. Bars express SD of two independent experiments. Panel B shows the fraction of cells with cell surface expression of the TRAIL-R2 receptor as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares).

.

Discussion

It had been suggested by several investigators that radiation may lead to an enhancement of TRAIL-toxicity and as such this combined modality may have therapeutic potential. [6-12,14] In most cases, the effect of radiation and TRAIL on cell survival had been only investigated using short term viability assays (crystal violet, MTT). [6-11,14] However, these assays do not take into account (later) cell death e.g. due to mitotic catastrophy of cells that did not undergo apoptosis initially. As we show here for radiation, such can lead to a dramatic underestimation of ultimate loss of reproductive capacity of cells (the ultimate goal in cancer therapy) as it is detected by clonogenic assay. Similar data were reported in a recent study by Nagane et al. [14]. In their study, a synergistic effect on apoptosis (TUNEL assay) by the combination therapy was found in 2 cell lines (T98G and U251). However, in only one cell line (T98G) this translated into some synergy when evaluated by the clonogenic assay. In the other cell line (U251) only additivity was found for this endpoint, like what we show here for the A172 cells. Therefore short term assays may be mis-interpreted in terms of synergy if certain treatments lead to accelerated (apoptotic) cell death without having an effect of the extent of ultimate cell death.

In our current study, we tried to test whether radiation would also synergistically interact with TRAIL in inducing cell death in a glioblastoma cell line A172 in order to bypass the typical radio-resistance of GBM tumor cells. Although, we found borderline significant synergy for early cell death induction (apoptosis, MTT), as was found by many others [6-12,14] no evidence was found to support the idea that the combination treatment enhanced the extent of ultimate killing (clonogenic assay) of radio-resistant A172 cells. Rather, the treatments were additive.

A possible explanation of synergistic apoptosis induction after combination treatment, which is frequently stated in the literature, could be radiation-induced upregulation of TRAIL receptors, thereby enhancing receptor modulated rhTRAIL killing [7,14,15]. In this study, no radiation-induced upregulation of membrane bound TRAIL-R1/R2 receptors in A172 cells was detected. Nor was a detectable increase found in the fraction of cells that expressed these receptors at their surface. This may explain why we only found borderline synergy for early death induction. The fact that still some synergy is found may indicate that radiation triggers some signaling pathways that facilitate TRAIL-induced apoptosis or vice versa. In any case, the effect we found in glioma cells was minor and consistent with Ciusani et al. who also found no up-regulation of TRAIL receptors (FACS analysis) after ionizing radiation (2 Gy) on glioma cell lines [12]. Yet, Nagane et al. did find up-regulation of TRAIL-R2 protein levels after radiation in some of the glioma cell lines investigated [14]. However, they did not evaluate whether this also lead to increased membrane receptor expression. So unlike other cell lines such as acute T lymphoblastic leukemia (MOLT-4) cells [11] and colorectal carcinoma (Colo205; HCT-15), lung (NCI-H460)[16] and breast carcinoma cells (MCF7)[10], several glioma cells may be radiation

resistant in terms of up-regulating these receptors and this may in fact contribute to a reduced is situ tumor radio-resistance on top of their intrinsic cellular radio-resistance.

Besides the conclusion that glioma cells may not show (much) synergy between radiation and TRAIL, our data also argue for re-evaluation of the observed synergy between these modalities seen in rapid death endpoint assays in other cell lines [6-12]. In none of these studies the efficacy of TRAIL and radiation was determined used a clonogenic assay. In fact, our finding that the extent of TRAIL-induced clonogenic death exceeded the extent of rapid apoptotic cell death underscores the need for such re-evaluation.

Finally, our findings demonstrate that glioma cells do express TRAIL receptors at their cell surface and are sensitive to TRAIL-induced cell death. This implies that glioma radio-resistance may not be associated with a cross-resistance to TRAIL. Hence, our study does not exclude rhTRAIL treatment as an adjuvant therapy in the treatment of patients with a malignant brain tumor. However, although only one glioma cell line was studied here, our data combined with Nagane et al. [14] suggest that a synergistic interaction between the modalities is not to be expected for several gliomas.

References

1. Kuijlen JM, Mooij JJ, Platteel I, Hoving EW, van der Graaf WT, Span MM, Hollema H, den Dunnen WF: TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. J Neurooncol .78: 161-171, 2006
2. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH: Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 104: 155-162, 1999
3. Roth W, Isenmann S, Naumann U, Kugler S, Bahr M, Dichgans J, Ashkenazi A, Weller M: Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. Biochem Biophys Res Commun 265: 479-483, 1999
4. Knight MJ, Riffkin CD, Muscat AM, Ashley DM, Hawkins CJ: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. Oncogene 20: 5789-5798, 2001
5. Rohn TA, Wagenknecht B, Roth W, Naumann U, Gulbins E, Krammer PH, Walczak H, Weller M: CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. Oncogene 20: 4128-4137, 2001
6. Zhou Q, Fukushima P, DeGraff W, Mitchell JB, Stetler SM, Ashkenazi A, Steeg PS: Radiation and the Apo2L/TRAIL apoptotic pathway preferentially inhibit the colonization of premalignant human breast cells overexpressing cyclin D1. Cancer Res 60: 2611-2615, 2000
7. Di Pietro R, Secchiero P, Rana R, Gibellini D, Visani G, Bemis K, Zamai L, Miscia S, Zauli G: Ionizing radiation sensitizes erythroleukemic cells but not normal erythroblasts to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)--mediated cytotoxicity by selective up-regulation of TRAIL-R1. Blood 97: 2596-2603, 2001
8. Kim MR, Lee JY, Park MT, Chun YJ, Jang YJ, Kang CM, Kim HS, Cho CK, Lee YS, Jeong HY, Lee SJ: Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. FEBS Lett 505: 179-184, 2001
9. Ramp U, Caliskan E, Mahotka C, Krieg A, Heikaus S, Gabbert HE, Gerharz CD: Apoptosis induction in renal cell carcinoma by TRAIL and gamma-radiation is impaired by deficient caspase-9 cleavage. Br J Cancer 88: 1800-1807, 2003
10. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 97: 1754-1759, 2000
11. Gong B, Almasan A: Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. Cancer Res 60: 5754-5760, 2000
12. Ciusani E, Croci D, Gelati M, Calatozzolo C, Sciacca F, Fumagalli L, Balzarotti M, Fariselli L, Boiardi A, Salmaggi A: In vitro effects of topotecan and ionizing radiation on TRAIL/Apo2L-mediated apoptosis in malignant glioma. J Neurooncol 71: 19-25, 2005
13. Wang L, Wei Q, Wang LE, Aldape KD, Cao Y, Okcu MF, Hess KR, El Zein R, Gilbert MR, Woo SY, Prabhu SS, Fuller GN, Bondy ML: Survival prediction in patients with glioblastoma multiforme by human telomerase genetic variation. J Clin Oncol 24: 1627-1632, 2006
14. Nagane M, Cavenee WK, Shiokawa Y: Synergistic cytotoxicity through the activation of multiple apoptosis pathways in human glioma cells induced by combined treatment with ionizing radiation and tumor necrosis factor-related apoptosis-inducing ligand. J Neurosurg 106: 407-416, 2007
15. Arizono Y, Yoshikawa H, Naganuma H, Hamada Y, Nakajima Y, Tasaka K: A mechanism

of resistance to TRAIL/Apo2L-induced apoptosis of newly established glioma cell line and sensitisation to TRAIL by genotoxic agents. Br J Cancer 88: 298-306, 2003
16. Marini P, Schmid A, Jendrossek V, Faltin H, Daniel PT, Budach W, Belka C: Irradiation specifically sensitises solid tumour cell lines to TRAIL mediated apoptosis. BMC Cancer 5:5.: 5-2005

Chapter 8

General discussion and future perspectives

The GBM is a highly aggressive tumor and shows major radio and chemo-resistance.

As radio-and chemotherapy exert their cell killing effect through the intrinsic "mitochondrial" apoptosis pathway of the GBM it seems logically that defects in the mitochondrial pathway may contribute to the intrinsic resistance of these conventional therapies. To bypass intrinsic resistance it is mandatory to evaluate the apoptosis inducing effect of drugs which can activate the "extrinsic" pathway of GBM cells, for example TRAIL [1, 8, 9, 13, 16, 18, 23]. The organized way of getting rid of malignant cells by apoptosis in combination with the lack of neuro- or systemic toxicity makes TRAIL an interesting molecule to treat the GBM acting through the extrinsic apoptosis inducing pathway [2, 28]. The applicability of TRAIL as apoptosis inducing agent has been extensively evaluated in several publications describing both in vitro and in vivo results (see chapter 2). On the other hand various tumor cell lines, including GBM cell lines, also show variable sensitivity to TRAIL [19, 30]. Thus both extrinsic as intrinsic resistance occurs in GBM cells. To overcome resistance TRAIL therapy could be combined with a variety of conventional and/or novel targeted therapies (chapter 2). In addition, to evaluate and develop TRAIL therapies insight must be given in receptor expression of the death inducing receptors and pathway defects occurring in GBM cells which contribute to the resistance of the GBM cell to TRAIL [22, 28].

Chapter 2 discusses the biology of TRAIL/TRAIL receptor signaling, focuses on the promises and pitfalls of recombinant TRAIL, as a therapeutic agent alone and in combinational therapeutic approaches, for GBM. Chapter 2 sums up various defects within the extrinsic pathway which can be "targeted" to partially restore sensitivity for TRAIL. The conclusion from this chapter is that the vast body of evidence from preclinical data indicates that the rational design of combinational TRAIL-based approaches with conventional as well as novel therapeutics may ultimately help to combat GBM. As TRAIL alone shows no major systemic toxicity it remains the question if after combinational treatment no systemic toxicity will occur [14]. Although animal in vivo studies with TRAIL en Temozolomide showed no systemic toxicity, we know from patients treated with radiotherapy and Temozolomide that approximately 7% of these patients show grade 3 to 4 hematological disturbances leading to cessation of the therapy [30]. Other novel drugs such as Bortezomib and rapamycine show in vitro favorable death inducing effects on tumor cells but might exert toxic effects in the human in vivo situation. It has been shown that bortezomib in combination with rhTRAIL induced hepatotoxicity in in vitro experiments [20]. A therapeutic window could be detected for cell lines other than GBM lines. In contrast to other tumors it might be difficult for intracranial gliomas, which are relatively surrounded by an intact blood brain barrier, to find the right therapeutic window.

The aim of the study in Chapter 3 was to quantify the expression of the death receptors TRAIL-R1, TRAIL-R2 on primary GBM tumor specimens, with immunohistochemistry and RT-PCR. Immunohistochemistry showed that TRAIL-R2 was predominantly expressed on GBM cells, however the percentage of GBM cells expressing TRAIL-R2 was relatively

low, around 30%. In comparison TRAIL-R1 was only expressed around 19%. Interesting is the fact that TRAIL-R1 expression on the mRNA level has an inverse relation with the grading of gliomas, this was not seen with TRAL-R2. Through down regulation of TRAIL-R1 on both the protein and receptor level high grade glioma tumor cells might escape apoptosis induction from TRAIL-TRAIL receptor interaction. As a GBM tumor expresses more TRAIL-R2 it seems logically that "targeted" therapy directed against TRAIL receptors is focused on the TRAIL-R2 receptor. A recent study confirms our findings that the TRAIL-R2 receptor is the predominant TRAIL receptor in glioma cells and that TRAIL-R1 is of minor importance [27]. It has been shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis [25, 31]. TRAIL-R2 appears to signal apoptosis only after efficient receptor crosslinking by either native membranous TRAIL, aggregated sTRAIL variants, or by TRAIL preparations secondarily crosslinked by antibodies. Apoptosis signaling by TRAIL-R1 appears to be relatively independent of the receptor crosslinking characteristics of a particular form of TRAIL. Furthermore, it was shown that TRAIL-R2 had superior binding affinity for TRAIL, resulting in predominant binding of TRAIL to TRAIL-R2 over TRAIL-R1. These findings emphasize the rationale of "targeting" TRAIL to GBM cells [3, 5]. Therefore to fully exploit the therapeutic potential of TRAIL, a number of characteristics of both the TRAIL receptor system and TRAIL should be taken into account. For example the fact of widespread expression of the various TRAIL receptors throughout the human body; the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAILR2 and the solution behavior of particular TRAIL preparations are all important factors to deal with. On basis of the above mentioned essentials a novel fusion protein (scFvC54:sTRAIL) was developed (Chapter 4) with enhanced tumor selective apoptosis induction toward EGP2-positive tumor cells [4]. The characteristics of scFvC54:sTRAIL showed enhanced apoptosis inducing capacity by activating the TRAIL-R2 receptor through crosslinking. Furthermore, the fusion protein showed the potential to induce apoptosis in a monocellular way but more interesting also in a bicellular way in which specific binding to one cell results in the crosslinking of TRAIL receptors on a neighbouring tumor cell. After the development of the scFvC54:sTRAIL and the demonstration that the concept of "targeting" TRAIL to membranous bound antigens resulted in enhanced apoptosis inducing effects, various other fusion proteins were developed. Another fusion protein which was developed (scFv425:sTRAIL) had specificity for the EGF receptor. As GBM cells over express the EGF receptor, the scFv425:sTRAIL fusion protein can be used in the treatment of GBM. This fusion protein simultaneously blocks EGFR signaling; thereby sensitizing tumour cells to apoptosis, and induces apoptosis via TRAIL receptor signaling. This fusion protein efficiently activates apoptosis and shows promising in vivo activity [6]. This novel scFv425:sTRAIL fusion protein was subject of research in Chapter 5 and 6. First we evaluated the in vitro apoptotic inducing effects of scFv425:sTRAIL on various cell lines including GBM cells. A positive linear correlation between scFv425:sTRAIL binding and EGFR expression was found (Chapter 6). Not surprisingly, no correlation was found between the amount of EGFR, TRAIL-R1 or TRAIL-R2 expression and toxicity.

This is in concordance with the literature, and underscores the theory that the amount of apoptosis inducing effects of TRAIL is not limited by the numeric expression of membranous receptors but is more likely dependent on intracellular resistance effects.

The focus of Chapters 5 and 6 was on the delivery of the scFv425:sTRAIL fusion protein, since delivery might hamper the use of TRAIL or TRAIL fusion proteins in clinical use. It has been shown that only marginal amounts of TRAIL bypass the blood brain barrier (BBB) after systemic application. The question arises whether other delivery methods might be more efficient in delivering TRAIL to the tumor. In Chapter 5 a microencapsulation technique was evaluated for continuously delivery of TRAIL. The result of this study showed that CHO-K1 cells transfected with the gene encoding for the scFv425:sTRAIL protein can, after encapsulation in alginate capsules, produce stable scFv425:sTRAIL proteins. Also biocompatibility of the alginate capsules in mice brains was found suggesting no major immunological reaction towards these capsules. This study also demonstrated that producer cells could maintain normal growth capacity in capsules made of both intermediate and high-G alginates. Also the death inducing capacity of the produced fusion proteins was preserved. A potential drawback of the microencapsulation technique is the development of necrosis of the TRAIL producing cells between 16 and 30 days after encapsulation, due to growth expansion of the producer cells and the subsequent shortage of nutrients.

One major question remains to be answered. Can the microencapsulated TRAIL producer cells construct enough fusion proteins to deliver a toxic concentration of TRAIL in the vicinity of the tumor? As we know from diffusion techniques in general that the penetration of drugs into the brain will be only a few millimeters. As the microencapsulation delivery method depends on diffusion this might hinder its clinical applicability, unless many capsules can be implanted in and around the tumor remnants.

In Chapter 6 we therefore evaluated if the CED technique which is based on convection of drugs could be of interest for delivery of TRAIL to an intracerebral tumor. The aim of this study was to evaluate the efficacy of a single chain (scFv425):sTRAIL fusion protein with specificity for the EGF receptor in a variety of human cell lines and in an animal brain tumor model. Although the binding of the scFv425:sTRAIL fusion protein to the SW948 was relatively low (in comparison to some of the other cell lines), the sensitivity of SW948 cells to scFv425:sTRAIL proved to be extremely high. SW948 colon cancer cells were selected to be stereotactically xenografted in the cerebrum of SCID mice. Using this procedure over 90% of the injected mice developed SW948 xenografts within 14 days and seemed to be a excellent brain tumor model to investigate the scFv425:sTRAL antitumor activity. We chose to evaluate xenograft acceptance and antitumor activity by performing pre and post treatment MRI's. By performing a pre-treatment scan we could actually demonstrate uniform tumor growth with low variance and therefore we needed fewer animals for this study in comparison with studies evaluating the effect

of anticancer treatment by measuring the survival period of the animal. Disappointing was the conclusion that under the experimental conditions described in this manuscript, convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps appears to be insufficient to inhibit tumor growth. The limiting factor was the concentration of the scFv425:sTRAIL used in this study. Other methods of delivery of TRAIL, for GBM, are under investigation such as delivery of TRAIL by TRAIL producing stem cells [15, 17, 21, 24, 35]. Interestingly, systematic delivery of mesenchymal stem cells which produced TRAIL could prolong the survival of brainstem glioma-bearing mice, therefore mesenchymal stem cells may be an effective vehicle for the targeted delivery of therapeutic agents to gliomas [35].

As mentioned previously GBM cells exhibit intrinsic and extrinsic resistance for (non) conventional therapies. Numerous in vitro studies have demonstrated a variance in sensitivity of GBM cell lines for TRAIL or chemo/radiotherapy. It is known from human in vivo studies that various chemotherapeutics, used as monotherapy or in combination with radiotherapy do not lengthen overall survival [10, 12, 32, 33]. The only exception is Temozolomide in combination with radiotherapy [30]. In general the treatment strategy for GBM is surgery with radiotherapy. Temozolomide is only given when the patient meets certain criteria, such as a good performance score. Therefore it seems logical to evaluate possible synergistic apoptosis inducing effects of combination therapy with RT and TRAIL. In chapter 7 the combined death inducing effects of conventional radiotherapy and recombinant human TRAIL (rhTRAIL) were evaluated. We tried to test whether radiation would synergistically interact with TRAIL in inducing cell death in a glioblastoma cell line A172 in order to bypass the typical radioresistance of GBM tumor cells. Although, we found borderline significant synergy for early cell death induction (MTT), as was found by many others [8, 9, 11, 16, 18, 26, 29, 36] no evidence was found to support the idea that the combination treatment enhanced the extent of ultimate killing (clonogenic assay) of radioresistant A172 cells. Rather, the treatments were additive. Besides the conclusion that glioma cells may not show (much) synergy between radiation and TRAIL, our data also argue for re-evaluation of the observed synergy between these modalities seen in rapid death endpoint assays in other cell lines. In fact, our finding that the extent of TRAIL-induced clonogenic death exceeded the extent of rapid apoptotic cell death underscores the need for such re-evaluation. However, although only one glioma cell line was studied here, our data combined with Nagane et al. [26] suggest that a synergistic interaction between the rhTRAL and radiotherapy is not to be expected for the GBM.

Future perspectives

Targeting aspects

In this thesis a novel targeted TRAIL fusion protein was tested with specificity for the EGP2 receptor which is present in colorectal cancer and lung tumors,. It has been shown in the literature that the production of such targeted TRAIL fusion proteins is possible, and results in a drug with superior characteristics concerning its apoptosis inducing capacity compared to TRAIL alone. So, in parallel to the above, for GBM treatment with TRAIL, high effectiveness might be found in the use of a TRAIL fusion protein directed against a GBM specific membrane antibody like the EGF receptor (scFv425:sTRAIL) or its variants (mutants) that are even more specific for GBM (the EGFvIII receptor).

Both fusion proteins have been recombinantly manufactured and are available for in vitro research and could be used to evaluate their apoptosis effect on glioblastoma cells. Besides targeting specific receptors like EGP2 and EGFR, one might also raise effectiveness of TRAIL fusion products by trying to address the most specific TRAIL receptors. Recently a novel fusion protein scFv425:sTRAILmR1-5 was designed with specificity for the TRAIL-R1 receptor. In vitro, it showed superior apoptosis inducing activity in comparison with scFv425:sTRAIL in various cell lines including glioma cell lines. Since TRAIL-R1 has a different activation profile in respect toTRAIL-R2 and TRAIL-R1 is present on GBM cells (chapter 3) it would be interesting to evaluate the effect of this novel fusion protein in a broader GBM cell panel with and without adjuvant therapies such as RT and Temozolomide.

Other targets for the treatment of GBM with TRAIL and its possible fusion products might be the VEGF receptor. Although this receptor is not unique for GBM vasculature it is abundantly present there, as well as on most of the tumour cells themselves. Apoptosis induction by a anti-VEGFR-TRAIL fusion protein could be the result of a direct effect on the tumour cells or an indirect effect on the tumour vasculature. The toxicity profile of such anti-VEGFR-TRAIL fusion protein has to be established first, in vitro and in animal studies. This approach is different from toxicity of a product directly related with tumor cell kill, since endothelium within the heterogeneity of a GBM might behave differently form isolated endothelial cell lines. Mixed cell cultures and /or GBM spheroids may be the best models for such tests but are difficult to handle. Even better might be a genetically engineered GBM mouse model. As tumors are heterogeneous in nature and represent a mixture of multiple cell types (eg endothelium) changes in the tumor microenvironment have been shown to have a critical influence in the initiation and progression of tumors. These important characteristics are ultimately lost when cellular signalling is studied in vitro. Some of these limitations can be overcome by growing cancer cells in 3D cell culture systems. It is thus important to extend analysis to the study of tumors, both from murine xenograft models and genetically engineered mouse models of GBM.

Resistance aspects

It has been discussed previously that resistance against TRAIL is an topic which needs to be explored in order to find biological solutions to overcome this resistance and thereby optimizing TRAIL therapy. One way to overcome or by-pass resistance is to design multimodality treatment paradigms, since monotherapies will fail too easily by the activation of resistance pathways. Another way is to look more in detail to what is known about the resistance mechanisms. In GBM, EGFR and other tyrosine kinase receptor signalling pathways are well known aberrant pathways that contribute to this resistance against TRAIL induced apoptosis. Maybe that today's popular tyrosine kinase inhibitors (TKIs) can have a role in a multimodality attack against the tumor, circumventing the resistance pathways. Some of these kinase inhibitors, even used as monotherapy, have shown promising results in preclinical trials. It remains to be seen whether they will perform well in the clinic. Phase II clinical trials with EGFR-TKIs (ZD-1839, gefitinib; OSI-774, erlotinib) demonstrated some responses, however it is much more interesting, in the light of the above reasoning, to see whether a combination treatment of a TKI with (targeted) TRAIL shows more therapeutic effect.

Another interesting pathway to attack in combination with TRAIL inducing apoptosis is the so called cell survival pathway, the PKB/mTOR pathway. By hitting PI3K from the PKB/mTOR-pathway GBM cell survival can be limited, supporting the effect of other anti GBM drugs. A recent study argues for combining PI3K inhibitors (LY294002) with TRAIL receptor agonists or conventional chemotherapeutic agents in order to "prime" glioblastoma cells for death receptor– or chemotherapy-induced cell death.

In the field of combinations with "pathway inhibitors" the possible value of mTOR inhibitors should be mentioned as well. One of these, Everolimus®, is already on the market as second line chemotherapy for patients with renal cell cancer, and seems worthwhile to explore for GBM treatment in combination with a TRAIL fusion protein.

Radiation

Although Radiation therapy has been proven effective against GBM since many years – and has become "standard therapy" after or instead of surgery – the effectiveness is limited, because of "radioresistance". Such resistance might be overcome by a multimodality attack combining radiation therapy with (targeted) therapies. In chapter 7 the effect of combined γ-radiation-TRAIL therapy in a glioblastoma cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints was explored. The clonogenic results did not show a synergistic effect in contrast to the early apoptotic death assay which suggested synergistic activity. It should be mentioned here again that when short term assays are used to detect possible enhancing effects of combination therapies, they

may lead to inappropriate conclusions about synergy. Only clonogenic assays or isobolographic calculations give trustful clues about the existence of synergy, only additional effects or none of these.

Addressing the issue of the combination therapy of irradiation and TRAIL it might be concluded so far that combining both therapies will lead to more cell kill on the short term; long term prognosis, however, seems not to be influenced. To lengthen overall survival the addition of chemotherapy and /or pathway inhibitors, mentioned above, on top of radiation and TRAIL must be investigated.

Delivery

The ability to bypass tumor cell resistance for TRAIL or other therapies is one hurdle to take but the delivery of large drugs in general to the site of the tumor is another one. Targeted TRAIL therapy can only be successful if the TRAIL fusion protein is delivered to the site of the tumor and especially its infiltrating zone. The Convection Enhanced Delivery (CED) technique (chapter 6) and the alginate microencapsulation method (chapter 5) of TRAIL fusion proteins and other novel therapeutics could be helpful in achieving this goal. The next step in analyzing the feasibility of alginate encapsulated (scFv:sTRAIL protein) producer cells in treating brain tumors is to implant encapsulated producer cells in the brain of mice with and without an intracerebral tumor. Diffusion of the protein within the tumor, mass effect of the intracerebral lesion, edema surrounding the tumor, brain compliance and cerebrospinal fluid flow will be factors influencing the in vivo efficacy of the micro encapsulation method and must be analyzed before any prediction can be made if the microencapsulation method will be a future therapeuric modality in the treatment GBM. Also the efficacy of CED of scFv425:sTRAIL in the brain of mice that were stereotactically xenografted with EGFR-positive tumour cells was investigated. Under the experimental conditions described in this manuscript (chapter 6), convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps appeared to be insufficient to inhibit tumour growth. In our current model loading of the osmotic pump with higher concentrations of scFv425:sTRAIL, using pumps with higher output capacity, as well as starting the treatment at lower initial tumour burden may be necessary to evaluate the anti-tumour efficacy of this novel approach.

Stem cells

Several studies have demonstrated the existence of cancer stem cell (CSC)s in solid tumors, including GBM. It is now generally assumed that these CSC in particular have the ability to reinitiate tumor cell proliferation and that they form the basis for chemo- and radioresistance. CSC's today form the new major target for experiments concerning

the eradication of cancer and this holds true for GBM as well. Therefore, it is extremely worthwhile to test the effectiveness of TRAIL and it variants (fusion proteins) against these CSC's. This can be done by engineering human mesechymal stemcells (MSC) to express secretable-TRAIL. It was demonstrated that MSC could be used as delivery vehicles for sTRAIL targeting glioblastoma stemcells (GBSC) in vivo resulting in in vivo antitumor activity. The most prominent membrane "receptor" in GBM CSC's seems to be CD133. Therefore, the first step should be to produce an antiCD133 fusion TRAIL protein and look for its antitumor effect.

Conclusion

We can conclude that ample in vitro and in vivo studies have shown that TRAIL is efficient in killing tumor cells with no or minimal systemic toxicity. Furthermore, the design of TRAIL-based therapy combined with other modalities, thereby augmenting apoptosis induction or overcoming resistance is a well proven approach and should also be applied in future therapeutic strategies for patients with GBM.

Although in vitro and in vivo studies show promising results the only way to find out if systemic application of TRAIL, its variants or TRAIL receptor blocking antibodies, is feasible and safe, is through the initiation of a phase I/II study for GBM patients. According to a large number of studies and the stimulating results, Genentech and Amgen have launched phase II clinical trails in non-Hodgkin's lymphoma and non-small-cell lung carcinomas with rhTRAIL [34]. Also phase I trials with anti-DR5/anti-DR4 antibodies have been initiated and show safe toxicity profiles [7, 28, 34]. It's time to make full use of the TRAIL-TRAIL receptor interaction in GBM tumors, we cannot stay behind.

References

1. Arizono Y, Yoshikawa H, Naganuma H, Hamada Y, Nakajima Y, Tasaka K: A mechanism of resistance to TRAIL/Apo2L-induced apoptosis of newly established glioma cell line and sensitisation to TRAIL by genotoxic agents. Br J Cancer 88:298-306, 2003
2. Ashkenazi A: Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev 19:325-331, 2008
3. Bremer E, de BM, Samplonius DF, Bijma T, ten CB, de Leij LF, et al: Targeted delivery of a designed sTRAIL mutant results in superior apoptotic activity towards EGFR-positive tumor cells. J Mol Med 86:909-924, 2008
4. Bremer E, Kuijlen J, Samplonius D, Walczak H, de Leij L, Helfrich W: Target cell-restricted and -enhanced apoptosis induction by a scFv:STRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. Int J Cancer 109:281-290, 2004
5. Bremer E, Samplonius DF, van Genne L, Dijkstra MH, Kroesen BJ, de Leij LF, et al: Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:STRAIL fusion protein with specificity for human EGFR. J Biol Chem 280:10025-10033, 2005
6. Bremer E, van Dam GM, de Bruyn M, van Riezen M, Dijkstra M, Kamps G, et al: Potent systemic anticancer activity of adenovirally expressed EGFR-selective TRAIL fusion protein. Mol Ther 16:1919-1926, 2008
7. C.H.Mom, I.A.Sloots, S.de Jong, J.A.Gietema, E.G.E.de Vries, S.Sleijfer: TRAIL modulators, in J-C Soria: Targeted Therapies in Oncology, 2007, pp 207
8. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, et al: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 97:1754-1759, 2000
9. Ciusani E, Croci D, Gelati M, Calatozzolo C, Sciacca F, Fumagalli L, et al: In vitro effects of topotecan and ionizing radiation on TRAIL/Apo2L-mediated apoptosis in malignant glioma. J Neurooncol 71:19-25, 2005
10. DeAngelis LM: Brain tumors. N Engl J Med 344:114-123, 2001
11. Di Pietro R, Secchiero P, Rana R, Gibellini D, Visani G, Bemis K, et al: Ionizing radiation sensitizes erythroleukemic cells but not normal erythroblasts to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)--mediated cytotoxicity by selective up-regulation of TRAIL-R1. Blood 97:2596-2603, 2001
12. Fine HA, Dear KB, Loeffler JS, Black PM, Canellos GP: Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. Cancer 71:2585-2597, 1993
13. Finnberg N, Gruber JJ, Fei P, Rudolph D, Bric A, Kim SH, et al: DR5 knockout mice are compromised in radiation-induced apoptosis. Mol Cell Biol 25:2000-2013, 2005
14. Ganten TM, Koschny R, Sykora J, Schulze-Bergkamen H, Buchler P, Haas TL, et al: Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. Clin Cancer Res 12:2640-2646, 2006
15. Germano IM, Uzzaman M, Benveniste RJ, Zaurova M, Keller G: Apoptosis in human glioblastoma cells produced using embryonic stem cell-derived astrocytes expressing tumor necrosis factor-related apoptosis-inducing ligand. J Neurosurg 105:88-95, 2006
16. Gong B, Almasan A: Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. Cancer Res 60:5754-5760, 2000
17. Hingtgen S, Ren X, Terwilliger E, Classon M, Weissleder R, Shah K: Targeting multiple pathways in gliomas with stem cell and viral delivered S-TRAIL and temozolomide. Mol Cancer Ther 7:3575-3585, 2008
18. Kim MR, Lee JY, Park MT, Chun YJ, Jang YJ, Kang CM, et al: Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. FEBS Lett 505:179-184, 2001

19. Knight MJ, Riffkin CD, Muscat AM, Ashley DM, Hawkins CJ: Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. Oncogene 20:5789-5798, 2001
20. Koschny R, Ganten TM, Sykora J, Haas TL, Sprick MR, Kolb A, et al: TRAIL/bortezomib cotreatment is potentially hepatotoxic but induces cancer-specific apoptosis within a therapeutic window. Hepatology 45:649-658, 2007
21. Kosztowski T, Zaidi HA, Quinones-Hinojosa A: Applications of neural and mesenchymal stem cells in the treatment of gliomas. Expert Rev Anticancer Ther 9:597-612, 2009
22. Kuijlen JM, Bremer E, Mooij JJ, den Dunnen WF, Helfrich W: Review: On TRAIL for malignant glioma therapy? Neuropathol Appl Neurobiol 36:168-182, 2010
23. Marini P, Jendrossek V, Durand E, Gruber C, Budach W, Belka C: Molecular requirements for the combined effects of TRAIL and ionising radiation. Radiother Oncol 68:189-198, 2003
24. Menon LG, Kelly K, Yang HW, Kim SK, Black PM, Carroll RS: Human bone marrow-derived mesenchymal stromal cells expressing S-TRAIL as a cellular delivery vehicle for human glioma therapy. Stem Cells 27:2320-2330, 2009
25. Muhlenbeck F, Schneider P, Bodmer JL, Schwenzer R, Hauser A, Schubert G, et al: The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J Biol Chem 275:32208-32213, 2000
26. Nagane M, Cavenee WK, Shiokawa Y: Synergistic cytotoxicity through the activation of multiple apoptosis pathways in human glioma cells induced by combined treatment with ionizing radiation and tumor necrosis factor-related apoptosis-inducing ligand. J Neurosurg 106:407-416, 2007
27. Nagane M, Shimizu S, Mori E, Kataoka S, Shiokawa Y: Predominant antitumor effects by fully human anti-TRAIL-receptor2 (DR5) monoclonal antibodies in human glioma cells in vitro and in vivo. Neuro Oncol 2010
28. Oldenhuis CN, Stegehuis JH, Walenkamp AM, de Jong S, de Vries EG: Targeting TRAIL death receptors. Curr Opin Pharmacol 8:433-439, 2008
29. Ramp U, Caliskan E, Mahotka C, Krieg A, Heikaus S, Gabbert HE, et al: Apoptosis induction in renal cell carcinoma by TRAIL and gamma-radiation is impaired by deficient caspase-9 cleavage. Br J Cancer 88:1800-1807, 2003
30. Stupp R, Mason WP, van dB, Weller M, Fisher B, Taphoorn MJ, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987-996, 2005
31. Wajant H, Moosmayer D, Wuest T, Bartke T, Gerlach E, Schonherr U, et al: Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 20:4101-4106, 2001
32. Walker MD, Alexander E,Jr, Hunt WE, MacCarty CS, Mahaley MS,Jr., Mealey J,Jr., et al: Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. J Neurosurg 49:333-343, 1978
33. Walker MD, Green SB, Byar DP, Alexander E,Jr, Batzdorf U, Brooks WH, et al: Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med 303:1323-1329, 1980
34. Wang S: The promise of cancer therapeutics targeting the TNF-related apoptosis-inducing ligand and TRAIL receptor pathway. Oncogene 27:6207-6215, 2008
35. Yang B, Wu X, Mao Y, Bao W, Gao L, Zhou P, et al: Dual-targeted antitumor effects against brainstem glioma by intravenous delivery of tumor necrosis factor-related, apoptosis-inducing, ligand-engineered human mesenchymal stem cells. Neurosurgery 65:610-24; discussion 624, 2009
36. Zhou Q, Fukushima P, DeGraff W, Mitchell JB, Stetler SM, Ashkenazi A, et al: Radiation and the Apo2L/TRAIL apoptotic pathway preferentially inhibit the colonization of premalignant human breast cells overexpressing cyclin D1. Cancer Res 60:2611-2615, 2000

Summary

Despite conventional treatment protocols (surgery, radiotherapy and chemotherapy), is the prognosis for patients with a glioblastoma, extremely poor. There is an urgency to develop new treatment strategies to improve outcome. Escapism of apoptosis, the intrinsic suicide program of the cell, is a hallmark of human cancer. Defects in apoptosis may confer resistance to therapy because most current treatment approaches initiate cell death through activation of an apoptosis pathway. Current attempts to improve the survival of glioblastoma patients will have to include strategies that specifically target tumor cell resistance to apoptosis. Of interest for GBM therapy is the selective induction of apoptosis using the pro-apoptotic tumor necrosis factor-related apoptosis inducing ligand (TRAIL).

TRAIL acts primarily through induction of the extrinsic apoptosis pathway in contrast to chemo and radiotherapy who act through the intrinsic mitochondrial pathway. This thesis elucidates TRAIL as a potential anti-neoplastic drug as TRAIL can bypass intrinsic (mitochondrial) resistance of tumor cells by acting its death inducing effect through the extrinsic pathway, evidently without causing systemic toxicity. Therefore TRAIL might be a new adjuvant therapy in the battle against GBM.

Chapter 1 outlines the aim of this thesis. **Chapter 2** presents a review which addresses several aspects of TRAIL-TRAIL receptor interaction and the potential of TRAIL as an anti-neoplastic agent in the treatment of malignant gliomas.

TRAIL receptor expression is found in many normal tissues and malignant cells of various origin. Preclinical studies show a variable sensitivity of tumor cells for TRAIL, including GBM cells, without major systemic-or neuro toxicity. Animal in vivo studies showed no major systemic toxicity after treatment with TRAIL. Phase 1 studies have investigated the applicability of rhTRAIL and TRAIL receptor antibodies in solid and hematological tumors (no GBM tumors) have been executed or are still in progress. In concordance with the preclinical studies, these trials showed (preliminary data) again that rhTRAIL and TRAIL receptor treatment is feasible without major systemic toxicity. Although systemic toxicity seems not to be a major obstacle for the introduction of TRAIL treatment for patients with a GBM, other restrictions can be. TRAIL induction of the apoptosis pathway can be restrained by intracellular resistance mechanisms. Several in vitro studies point out that combination therapy with various (non)conventional drugs and TRAIL overrides resistance of glioma cells to TRAIL. Yet, it is not known if these combination therapies do lead to induction of systemic- or neurotoxicity. As highlighted in this chapter, TRAIL can be combined with a variety of different conventional and novel therapeutic strategies to yield synergistic apoptotic activity. Of particular interest is the use of dual purpose TRAIL-based molecules, such as the EGFR-targeted TRAIL fusion protein scFv425:sTRAIL. This fusion protein simultaneously blocks EGFR signaling, thereby sensitizing tumor cells to apoptosis, and induces apoptosis via TRAIL receptor signaling. It has been shown in the literature that this

fusion protein efficiently activates apoptosis and shows promising in vivo activity. Chapter 2 also elaborates on other TRAIL- combination strategies which may help to optimize anti-GBM activity.

In **Chapter 3** we describe the amount of TRAIL-R1 and TRAIL-R2 expression on primary GBM specimens. A requirement for success of TRAIL therapy, in patients with a GBM, is the presence of TRAIL receptors on GBM cells. Studies investigating TRAIL receptor expression were mainly focused on homogeneic cell lines which differ from primary GBM tumor tissue. Therefore, quantification of TRAIL receptor expression in primary GBM cells was instigated. Furthermore a possible association between expression of TRAIL-R1/TRAIL-R2 and survival was evaluated.

It was concluded that TRAIL-R1 and TRAIL-R2 expression patterns on tumor cells are independent prognostic factors for survival in patients with a GBM. Both receptors could be targets for TRAIL therapy. However since TRAIL-R2 is more expressed, on GBM tumor cells than TRAIL-R1, TRAIL-R2 seems to be the most important target for future TRAIL therapy.

In **Chapter 4** the development, production and properties of a single chain –TRAIL fusion protein (scFvC54:sTRAIL) were described. The scFvC54:sTRAIL fusion protein was designed to induce apoptosis by cross-linking of agonistic TRAIL-R2 receptors only after specific binding of scFvC54:sTRAIL to the abundantly expressed carcinoma-associated cell surface antigen EGP2 (epithelial glycoprotein 2). Analysis of the solution of the scFvC54:sTRAIL showed soluble stable homogeneous trimers of scFvC54:sTRAIL with no or only minimal aggregate formation. This is important as certain aggregated TRAIL forms have shown organ-specific toxicity. When EGP2-positive tumor cells were subjected to treatment with scFvC54:sTRAIL, an efficient induction of apoptosis was observed thereby concluding that, after production, the biological functionality of the fusion protein was maintained. Interesting was the fact that the target antigen restricted apoptosis inducing capacity of scFvC54:sTRAIL was directly proportional to the degree of TRAIL-R2 receptor crosslinking. The conclusion of this chapter was that the scFvC54:sTRAIL fusion protein efficiently induced bi- or multi-cellular reciprocal apoptosis in a target antigen restricted fashion.

As discussed in the review article presented in Chapter 2, the method of drug delivery is an important factor for success in the drug treatment of GBM tumors. In **Chapter 5** we evaluated the alginate microencapsulation method. Chinese Hamster Ovary cells (CHO-K1) were engineered to produce the scFv425:sTRAIL protein. The CHO-K1 producer cells were encapsulated in an alginate capsule with a semi-permeable membrane through which the scFv425:sTRAIL protein could be released. Utilizing this method a constant production of fusion protein was enabled, thereby creating a biological scFv425:sTRAIL producing factory. The biological functionality of the apoptosis

inducing scFv425:sTRAIL protein, which was released through the microencapsulation method, was studied in vitro and analysis of the intracerebral biocompatibility of alginate capsules was performed by implantation of empty alginate capsules in the brain of mice. The study concluded that the biological functionality of the produced scFv425:sTRAIL protein was maintained and intracerebral biocompatibility of the capsules was warranted. The next step is to design an in vivo study with a rat or mouse brain tumor model with intracerebral application of alginate encapsulated scFv425:sTRAIL - producer cells and to evaluate the antitumor effects of this delivery method.

Previous publications showed that the scFv425:sTRAIL fusion protein resulted in enhanced apoptosis in cancer cells. These promising in vitro results justified in vivo experiments and in **Chapter 6** the in vivo efficacy of the scFv425:sTRAIL fusion protein in a mouse brain tumor model was investigated. A brain tumor model was developed by xenografting a SW948 cell line in the cerebrum of an SCID mouse. Since the scFv425:sTRAIL fusion protein induced a strong apoptotic signal in the SW948 cell line and xenografting of this cell line into the cerebrum of SCID mice resulted in a more than 90 % acceptance of the graft, this seemed a good animal model for testing the in vivo death inducing potential of the scFv425:sTRAIL fusion protein. Through the convection enhanced delivery method (CED), with an Alzet® osmotic pump, the scFv425:sTRAIL fusion protein or a placebo was intracerebrally, intratumorally infused. Pre and post treatment MRI was used to detect in vivo efficacy of the scFv425:sTRAIL fusion protein. Eventually, the in vivo efficacy of the scFv425:sTRAIL fusion protein, in the animal brain tumor model used in this study, could not be shown. After careful analysis it was concluded that the doses of the infused scFv425:sTRAIL was to low to exert a proper antitumor effect. New animal (CED) brain tumor experiments with gradual dose increment should be initiated to evaluate the minimal effective dose.

It has been brought up that radiation can enhance the apoptosis-inducing efficacy of the TNF Related Apoptosis Inducing Ligand (TRAIL). In **Chapter 7** we evaluated the effect of combined γ-radiation-TRAIL therapy in a glioblastoma (A172) cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints. Although the combination of radiation and TRAIL lead to a small synergy in apoptosis induction measured by short term assays, this did not translate to synergy in ultimate loss of clonogenicity, where radiation and TRAIL merely showed additive effects. Besides the conclusion that glioma cells may not show (much) synergy between radiation and TRAIL, our data also argue for re-evaluation of the observed synergy between these modalities seen in rapid death endpoint assays in other studies. In fact, our finding that the extent of TRAIL-induced clonogenic death exceeded the extent of rapid apoptotic cell death underscores the need for such re-evaluation. This study shows an additive effect on cell death after combination treatment of radiation and TRAIL and therefore TRAIL might have a place within the treatment of patients with a GBM.

It can be concluded that TRAIL and TRAIL deratives have unique properties and can play a role in the treatment of GBM. Multimodality treatment is the key factor for successful treatment of GBM. TRAIL in combination with (non) conventional treatment modalities must be explored. TRAIL-combination therapy might overcome resistance of GBM cells to chemo-radiotherapeutic approaches and hopefully prolonging overall survival for this patient group.

Nederlandse samenvatting (Summary in Dutch)

Ondanks conventionele behandelingsprotocollen (chirurgie, radiotherapie en chemotherapie) blijft de algemene prognose voor de patiëntengroep met een GBM zeer slecht. Het is daarom noodzakelijk om nieuwe behandelingsstrategieën te ontwikkelen die de overleving van deze patiëntengroep kan verlengen. Apoptose, ook wel geprogrammeerde celdood genoemd, is een intrinsieke eigenschap die iedere cel bezit en kan aanwenden om zichzelf uit te schakelen indien dit noodzakelijk is. Opvallend is dat tumorcellen intrinsieke mechanismen beschikbaar hebben die apoptose inductie kunnen inhiberen. Daardoor kunnen bestaande therapieën, die vooral via de intrinsieke apoptose route hun celdodend effect sorteren, worden geblokkeerd. Hierdoor ontstaat resistentie van tumorcellen voor de bestaande therapieën. Nieuw te ontwikkelen therapieën moeten in staat zijn om deze resistentie terug te dringen. Interessant als nieuw medicament voor GBM behandeling is de pro-apoptoptische ligand TRAIL (TNF Related Apoptosis Inducing Ligand). TRAIL induceert apoptose via de extrinsieke route in tegenstelling tot chemo en radiotherapie die via de intrinsieke mitochondriale route hun apoptose effect uitvoeren.

Dit proefschrift belicht TRAIL als potentieel anti-neoplastisch medicament dat de intrinsieke (mitochondriale) tumorcel resistentie kan omzeilen door apoptose te induceren via de extrinsieke apoptose route en waarbij klaarblijkelijk geen systemische toxiciteit ontstaat. Vanwege deze eigenschappen zou TRAIL een nieuwe adjuvante therapie kunnen worden in de strijd tegen het GBM.

Hoofdstuk 1 licht de onderzoeksvragen van dit proefschrift toe. In **hoofdstuk 2** wordt een overzichtsartikel gepresenteerd waarin diverse aspecten van TRAIL-TRAIL receptor interactie en het potentieel van TRAIL om als anti-kanker medicament voor GBM tumoren in aanmerking te komen, wordt belicht.

TRAIL receptor expressie is aanwezig op vele normale cellen en weefsels binnen ons lichaam. Tumorcellen van diverse origine vertonen ook TRAIL receptor expressie. Preklinische onderzoeken hebben een variabele sensitiviteit aangetoond van tumorcellen voor TRAIL (incluis GBM cellen), zonder evidente systemische of neurotoxiciteit. Uit verschillende dierexperimentele studies werd geconcludeerd dat er geen majeure systemische toxiciteit na behandeling met TRAIL aantoonbaar was. Fase 1 studies met rhTRAIL en anti-TRAIL receptor antilichamen in solide (geen GBM tumoren) en hematologische tumoren zijn lopende of uitgevoerd. In overeenstemming met de preklinische studies komen de klinische studies tot de conclusie dat rhTRAIL-TRAIL receptor behandeling mogelijk is zonder duidelijke systemische toxiciteit (voorlopige data). TRAIL toxiciteit lijkt dus niet een beperkend rol te spelen om TRAIL als antikanker medicament voor GBM patiënten te introduceren. Wat wel een beprekende rol kan spelen is de mogelijke resistentie van GBM cellen voor TRAIL. Diverse in vitro studies hebben aangetoond dat deze resistentie voor TRAIL overwonnen kan worden door TRAIL te combineren met (non) conventionele medicamenten of therapieën. Het is echter niet duidelijk of deze combinatie behandelingen wel zouden kunnen leiden tot systemische -of neurotoxiciteit.

Zoals beschreven in dit hoofdstuk kan TRAIL worden gecombineerd met conventionele en nieuwe therapeutische strategieën om daarmee versterkte apoptotische activiteit te induceren. In het kader van nieuwe therapeutische strategieën is het interessant om te kijken naar moleculen die een duale functie hebben. Het scFv425:sTRAIL fusie-eiwit, is een molecuul met een dubbel functie, enerzijds kan dit molecuul via de scFv425 zijde de EGF receptor (EGFR) binden en via de TRAIL zijde de TRAIL receptor binden. Op deze manier kan tegelijkertijd de EGFR geremd worden en de TRAIL receptor geactiveerd worden waardoor de cel extra geactiveerd wordt om in apoptose te gaan. Het is in de literatuur proefondervindelijk aangetoond dat deze strategie werkt en dat het fusie-eiwit efficiënt apoptose induceert zowel in vitro als in vivo. Hoofdstuk 2 gaat tevens verder in op diverse andere TRAIL combinatie strategieën die aangrijpen op zowel intrinsiek als extrinsieke cellulaire regelmechanismen en die kunnen bijdragen aan het bestrijden van het GBM.

In **hoofdstuk 3** beschrijven we de mate van expressie van TRAIL-R1 en TRAIL-R2 receptoren op primair glioblastoom weefsel. Wil TRAIL therapie succesvol zijn dan dienen TRAIL-R1 en TRAIL-R2 receptoren aanwezig te zijn op GBM cellen. De meeste studies die TRAIL receptor expressie hebben geanalyseerd hebben gebruik gemaakt van homogene cellijnen die duidelijk verschillen van primair GBM tumor weefsel. In dit hoofdstuk is de mate van TRAIL receptor expressie beoordeeld en tevens is geanalyseerd of de mate van TRAIL-R1 en TRAIL-R2 expressie geassocieerd kon worden met overleving. De conclusie was dat de mate van expressie van zowel TRAIL-R1 en TRAIL R2 receptoren een onafhankelijke prognostische factor was voor overleving. Beide receptoren kunnen targets zijn voor TRAIL therapie. Echter omdat TRAIL-R2 meer tot expressie wordt gebracht dan TRAIL-R1 lijkt TRAIL-R2 een meer geschikte kandidaat om als target te dienen voor toekomstige TRAIL therapie.

In **hoofdstuk 4** worden de ontwikkeling, productie en intrinsieke eigenschappen beschreven van een single chain-TRAIL fusie eiwit (scFvC54:sTRAIL). Het scFvC54:sTRAIL fusie-eiwit is ontworpen om apoptose te induceren door middel van het crosslinken van TRAIL-R2 receptoren wat alleen tot stand kwam na specifieke binding van het scFvC54-:sTRAIL fusie-eiwit aan het carcinoom geassocieerde oppervlakte antigeen EGP2 (Epithelial Glycoprotein 2).

Analyse van de geproduceerde oplossing met daarin het scFvC54:sTRAIL fusie eiwit toonde aan dat het fusie-eiwit geproduceerd kon worden zonder dat fusie-eiwit aggregaten ontstonden. Dit is belangrijk aangezien aangetoond is dat geproduceerde TRAIL aggregaten kunnen leiden tot weefsel toxiciteit. Wanneer EGP2 positieve tumorcellen werden blootgesteld aan het scFvC54:sTRAIL, kon apoptose inductie worden geconstateerd, wat aantoonde dat de biologische functionaliteit van het fusie-eiwit behouden bleef na productie. Interessant was het feit dat er een positieve relatie bestond tussen de mate van apoptose inductie door het scFvC54:sTRAIL fusie-eiwit en de hoeveelheid TRAIL-R2

receptor crosslinking. De conclusie van dit hoofdstuk was dat het scFvC54:sTRAIL fusie-eiwit efficiënt bicellulaire en/of multicellulaire apoptose induceert en dat dit op basis is van de specifieke “targeting” eigenschap van het fusie-eiwit.

In hoofdstuk 2 van dit proefschrift is al ingegaan op het feit dat bij de medicamenteuze behandeling van GBM tumoren de manier van toediening van een medicament van belang is voor de kans van slagen van de behandeling. **In hoofdstuk 5** evalueerden we de alginaat micro-encapsulatie techniek als methode van toediening. Chinese Hamster Ovary cellen (CHO-K1) werden zodanig gemanipuleerd dat ze het scFv425:sTRAIL fusie-eiwit konden produceren. Vervolgens werden deze CHO-K1 cellen geëncapsuleerd in een alginaat capsule met een semi-permeabel membraan waardoorheen het geproduceerde scFv425:sTRAIL kon worden afgegeven. Door middel van deze specifieke methode werd een constante productie en afgifte van scFv425:sTRAIL bewerkstelligd, waarbij een biologische scFv425:sTRAIL producerende fabriek werd gecreëerd. De biologische functionaliteit van het scFv425:sTRAIL fusie eiwit, na productie, werd in vitro getest. Tevens werd beoordeeld of lege alginaat capsules, die in een muizen brein waren geïmplanteerd, een intracerebrale immunologische reactie zouden opwekken. Het onderzoek toonde aan dat de functionaliteit van het geproduceerde fusie-eiwit in stand bleef en dat de alginaat capsules biocompatibel waren met de muizenhersenen. De volgende stap is om in een dierexperimenteel hersentumor model te evalueren of de alginaat micro-encapsulatie methode met scFv425:sTRAIL producerende cellen geschikt is om antitumor effecten te bewerkstelligen.

Eerdere publicaties toonden aan dat een nieuw ontwikkeld scFv425:sTRAIL fusie-eiwit versterkte apoptose inductie veroorzaakte in kanker cellen. Deze positieve in vitro bevindingen rechtvaardigden een in vivo onderzoek. In **hoofdstuk 6** onderzochten we de in vivo effectiviteit van het scFv425:sTRAIL fusie-eiwit in een muis hersentumor model. Een muis hersentumor model werd ontwikkeld door SW948 cellen aan te brengen in de hersenen van een SCID muis. Er werd gekozen voor dit model omdat SW948 cellen zeer sensitief waren voor het scFv425:sTRAIL fusie-eiwit en intracerebrale implantatie leidde to meer dan 90% graft acceptatie. Door middel van de convection enhanced delivery (CED) methode, met een Alzet® osmotische pomp werd het scFv425:sTRAIL of een placebo intracerebraal, intratumoraal geïnfundeerd. MRI scans van de muizen hersenen werden voor en na behandeling uitgevoerd ter beoordeling van de tumor grootte. De mate van tumor reductie was een maat voor effectiviteit van het scFv425:sTRAIL fusie-eiwit. Uiteindelijk kon, binnen het gekozen dierexperimentele model, geen evidente effectiviteit van het fusie-eiwit worden aangetoond. Na analyse van het resultaat kon worden aangetoond dat de totale dosis van het geïnfundeerd fusie-eiwit te laag was om anti-tumor effecten te bewerkstelligen. Nieuwe dierexperimenten, gebruik makend van het CED muis hersentumor model, waarbij de concentratie van het geïnfundeerde fusie-eiwit geleidelijk wordt verhoogd, moeten uitwijzen bij welke minimale dosis antitumor effecten worden waargenomen.

Het is gesuggereerd dat een combinatie van bestraling met TRAIL de apoptose inducerende werking van TRAIL kan versterken. **In hoofdstuk 7** werd het effect geanalyseerd van een combinatie behandeling van γ-radiotherapie en TRAIL op een A172 glioom cellijn. Hierbij werd zowel de vroeg apoptotische celdood als de clonogene overleving geëvalueerd. Hoewel de combinatie behandeling van bestraling en TRAIL een klein synergistisch effect vertoonde, gemeten met de korte termijn assay, vertaalde dit zich niet in een synergistisch effect gemeten m.b.v. de clonogene overlevingstest. Het combinatie effect gemeten m.b.v. een clonogene overlevingstest toonde geen synergie aan maar slechts additiviteit. Naast de conclusie dat er voor gliomen geen duidelijke synergistisch cel dodend effect ontstaat bij de combinatiebehandeling van bestraling en TRAIL pleiten onze data ook voor een herevaluatie van studies die een synergie hebben waargenomen, tussen deze twee modaliteiten, op basis van korte termijn assays. Het feit dat de mate van celdood, door TRAIL geïnduceerd, binnen de clonogene overlevingstest groter was dan gemeten door de korte termijn assay ondersteund mede ons pleidooi voor herevaluatie. Deze studie toont aan dat er een additief celdodend effect valt te verwachten van de combinatie behandeling van bestraling en TRAIL en er is dus een plaats voor TRAIL binnen de behandeling van patiënten met een GBM.

Geconcludeerd kan worden dat TRAIL en TRAIL varianten unieke eigenschappen hebben en daardoor een rol kunnen spelen bij de behandeling van het GBM. Een GBM behandeling met een combinatie van multiple modaliteiten van zowel conventionele als non-conventionele aard geeft de meeste kans op een succesvolle behandeling. TRAIL-combinatie therapie kan wellicht bijdragen aan het overwinnen van chemo-radiotherapeutische resistentie van het GBM en hopelijk de overleving van deze patiëntengroep verlengen.

Dankwoord

Diverse mensen binnen het UMCG en de RUG waren betrokken bij het creëren van een omgeving, waardoor het mogelijk was om de data en analyses te verkrijgen die in dit proefschrift zijn weergegeven. Allen zou ik hartelijk willen bedanken voor de vaak belangeloze inzet.

Beste Jan Jakob, in 1998 ben ik bij jou mijn opleiding tot neurochirurg begonnen. In 2000 ben ik onder jouw begeleiding gestart met neuro-oncologisch onderzoek. Het resultaat hiervan is beschreven in dit proefschrift.
Ik wil je ontzettend bedanken voor de intensieve begeleiding als promotor van dit proefschrift, maar ik wil je ook bedanken voor de tijd als opleider en de laatste jaren als collega. Ik heb veel van je geleerd en heb je altijd zeer gewaardeerd als mens en als collega. Sinds een aantal dagen ben je met pensioen en ik wens je, samen met Annelies, het allerbeste toe.

Lou de Leij wil ik bedanken voor het feit dat ik binnen zijn vakgroep twee jaar fulltime onderzoek heb mogen doen. Ik dank je voor de mogelijkheden die je me hebt geboden en voor je steun en betrokkenheid op de juiste momenten.

Wilfred, ik ben zeer verheugd dat je mijn copromotor bent. Je hebt een zeer groot aandeel gehad in dit proefschrift. In een voor mij belangrijke fase in mijn promotietraject, heb ik je om bijstand gevraagd. Jouw enthousiasme, je heldere analyses, het feit dat je altijd mogelijkheden zag voor publicaties heeft mij een boost gegeven. Wat ik het meest gewaardeerd heb was je oprechte inzet om samen met mij ervoor te zorgen dat er een “boekje” zou komen. Mijn dank hiervoor. Ik wens jou en Jane alle goeds toe.

Beste Wijnand, ik heb onder jouw begeleiding twee jaar lang fulltime basaal wetenschappelijk onderzoek verricht. Dit onderzoek vormde het fundament voor het proefschrift. Voor mij als clinicus, met een totaal andere opleidingsachtergrond, niet altijd even gemakkelijk maar zeker heel leerzaam. Dank voor je begeleiding.

Beste Paul en Bart. Jullie idee is uitgewerkt en de resultaten zijn beschreven in hoofdstuk 5. Die publicatie heeft het onderzoek weer schwung gegeven. Jullie bijdrage en die van één van jullie studenten, Jan-Freark de Boer, is belangrijk geweest voor mijn proefschrift. De manier waarop jullie je kennis en kunde combineren met die van andere, is een pluim waard. Bedankt voor jullie waardevolle aandeel en interesse in mijn onderzoek.

Beste Jelleke. Altijd interesse in mijn onderzoek en mijn familie. We hebben vele uurtjes naast elkaar gezeten in de kweekruimte en over van alles en nog wat gesproken. Van jou heb ik het cellen kweken en beoordelen geleerd. Je was altijd bereid om weer een batch voor me op te kweken. Het resultaat is er. Weer een proefschrift waar jij een bijdrage aan hebt geleverd. Dank hiervoor.

Beste Edwin en Douwe. In de twee jaar dat ik fulltime onderzoek deed hebben we nauw samengewerkt. Dank voor jullie bijdrage aan het bedenken en mee helpen uitvoeren van een aantal experimenten.

Verder wil ik alle medewerkers van het tumorimmunologie lab bedanken voor hun gastvrijheid en de mogelijkheden die ze me gegeven hebben om gebruik te kunnen maken van hun kennis en kunde.

Diverse studenten hebben een belangrijk aandeel gehad in mijn proefschrift.
Roelof Horn, dank voor jouw bijdrage aan het artikel, dat in hoofdstuk 5 staat beschreven. Jan-Freark de Boer, dank voor het uitvoeren van vele experimenten die in hoofdstuk 5 zijn beschreven. Wilma van Steenbergen, veel dank voor je inzet in de totstandkoming van de data en analyses die beschreven staan in hoofdstuk 7.

Beste Harrie, dank voor je persoonlijke bijdrage aan hoofdstuk 7, maar ook voor het feit dat we gebruik mochten maken van je lab en de hulp mochten inroepen van een aantal van jouw medewerkers die ons hebben geholpen met het verkrijgen van data voor hoofdstuk 7.

Beste Fred Zuiderveen, dank voor jouw bijdrage bij het verrichten van de dierexperimenten.

Maaike Disco en Aletta de Boer dank ik voor de opmaak en het ontwerp van mijn proefschrift.

Prof dr. H. Hollema, Prof dr. C.M.F. Dirven en Prof dr. M.J. Staal wil ik bedanken voor de bereidheid om dit proefschrift te beoordelen.

Het KWF wil ik bedanken voor het toekennen van een onderzoeksbeurs in 2000, zodat ik vrij van klinische taken onderzoek kon doen.

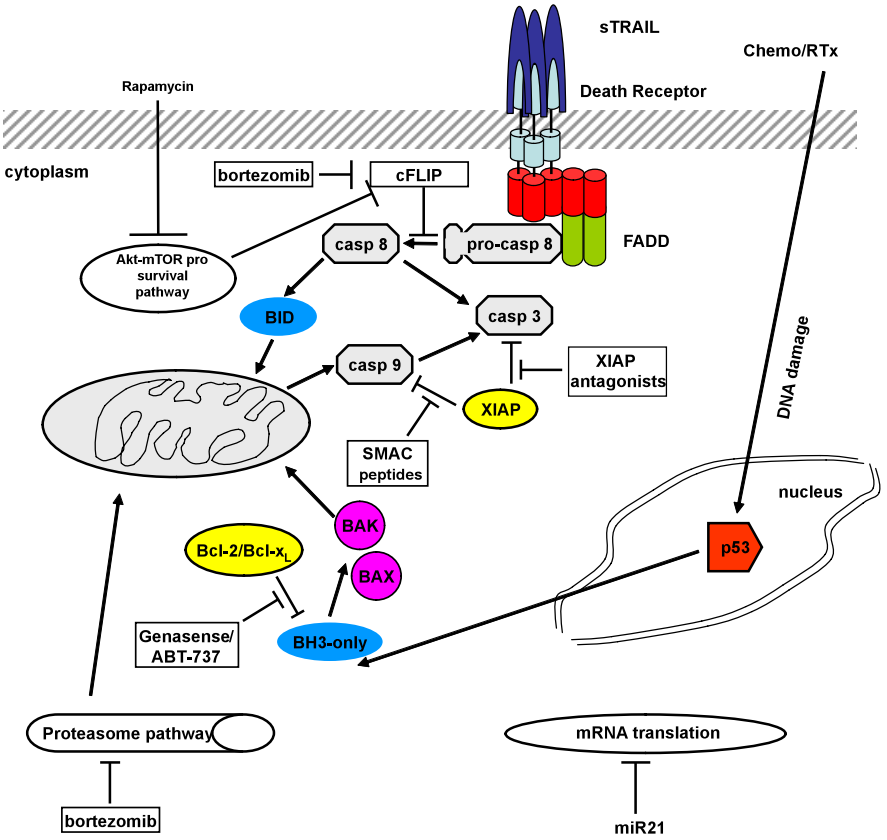
Beste Jan we zijn samen de opleiding tot neurochirurg gestart, hebben samen veel opgetrokken en zijn goede vrienden geworden. De klus is nu geklaard, tijd over, dus als je hulp nodig hebt bij het klussen hoor ik het graag. Volgend jaar weer bomen zagen? Bedankt dat je mijn paranimf wilde zijn.

Beste Michiel, aangezien we, in de gelukkige omstandigheid verkeerden om samen een kamer te delen konden we ook geregeld met elkaar van gedachten wisselen, ook over ons onderzoek. Ik wil jou bedanken voor je luisterend oor, je vriendschap en je collegialiteit. Ook jij bedankt dat je mijn paranimf wilde zijn. Ik wens je verder succes met het afmaken van jouw promotietraject.

Prof dr. K.G. Go, beste Gwan, indien we alles in historisch perspectief willen plaatsen dan moet vermeld worden dat jij de man van het eerste uur bent geweest. Samen met jou heb ik destijds een conceptaanvraag geschreven voor het KWF met als onderwerp immunotherapie. De aanvraag werd afgewezen en het onderwerp van onderzoek werd in de loop der tijd gewijzigd. Echter vanuit jouw initiële plan is er wel een ander onderzoeksvoorstel geschreven hetgeen uiteindelijk geresulteerd heeft in dit proefschrift. Dank voor de samenwerking.

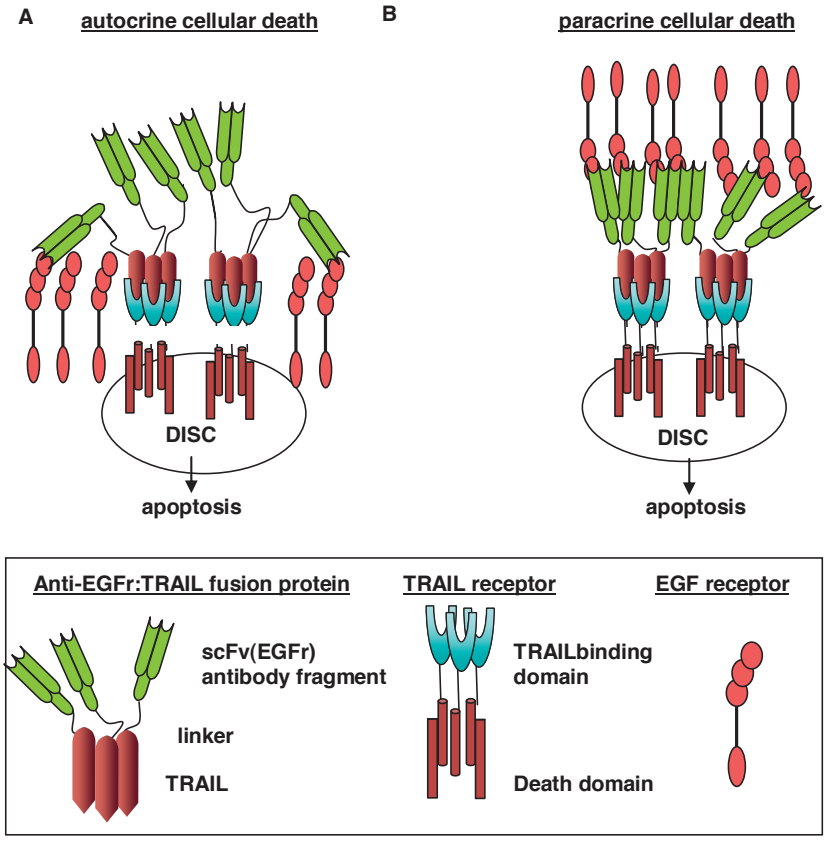
Collega stafleden, beste Eelco, Jan, Maarten, Marc, Michiel en Rob. Ik wil jullie allemaal bedanken voor de interesse die jullie hebben getoond in mijn onderzoek. Bedankt voor het waarnemen van mijn klinische taken op de momenten dat ik toch echt tijd moest besteden aan mijn onderzoek. Jullie flexibiliteit en collegialiteit is voor mij van groot belang geweest om dit proefschrift te kunnen afronden.

Figure 3



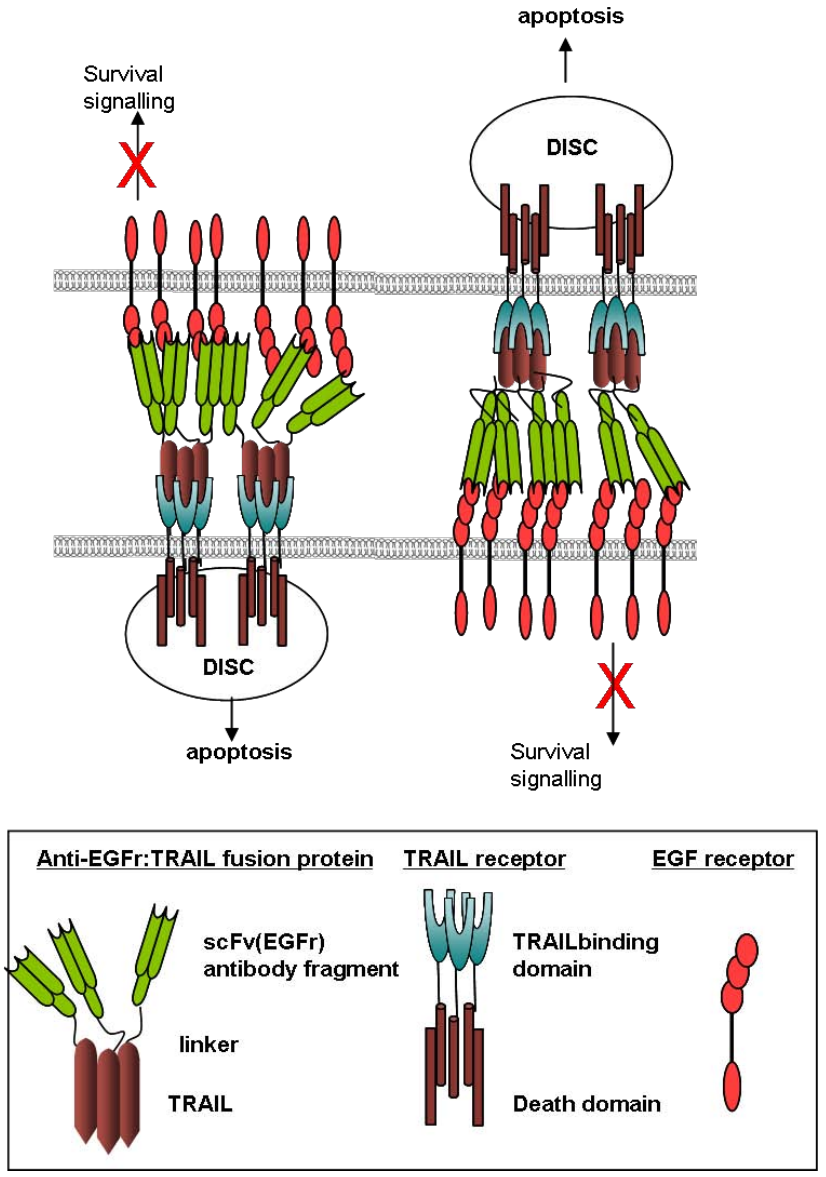
The two major apoptosis signaling pathways converge at the level of the effector caspases. Moreover, there can be cross-talk between the extrinsic and intrinsic pathways. Caspase 8 seems to be the key feature for this cross-talk. Caspase 8 cleaves BID, which in turn engages the mitochondria. leading to activation of caspase 9 and further activation of caspases 3, 6, and 7. Rapamycin, Bortezomib, miR21, XIAP antagonists, ATB737, SMAC peptides are all compounds having an inhibiting function on different parts of the extrinsic or intrinsic pathway and thereby preventing death inducing signalling (discussed within the review). (BCL2, B-cell chronic lymphocytic leukemia/lymphoma 2; BID: BH3-interacting domain death agonist; (BAK: BCL2 homologous antagonist/killer; BAX: BCL2-associated protein; XIAP: X-linked inhibitor of apoptosis protein; SMAC: second mitochondria-derived activator of caspase.

Figure 4



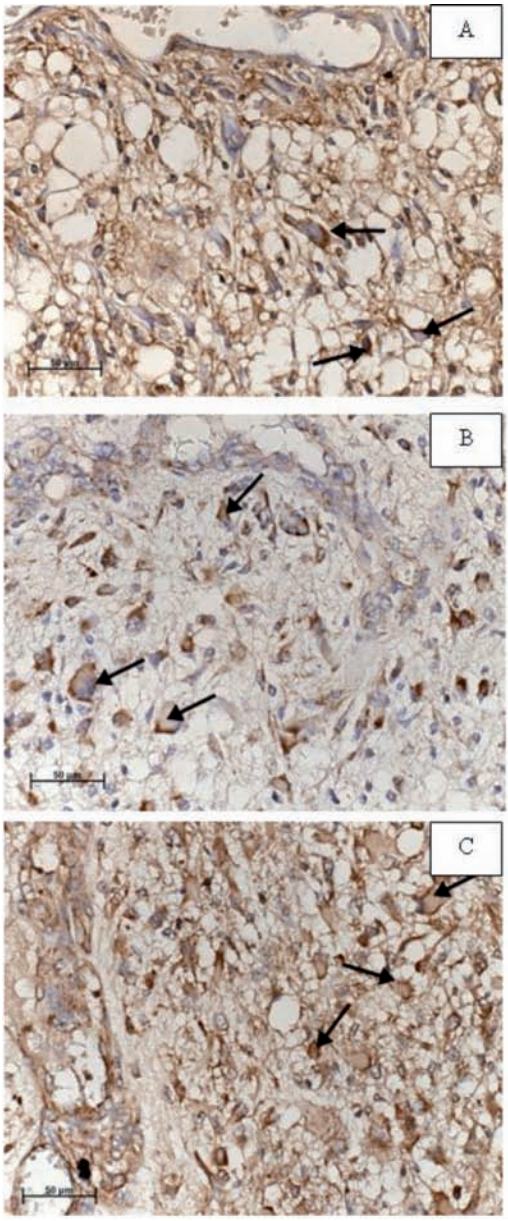
(A) ScFv:TRAIL ligand fusion protein binds via its antibody fragment [scFv(EGFR)] to its target antigen on a target antigen-positive tumour cells, which converts the soluble ligand to a fully active membrane-like ligand. Subsequently, this ligand binds to and cross-links its cognate receptors in an autocrine manner. (B) A scFv:TRAIL ligand fusion protein binds via its antibody fragment [scFv(EGFR)] to the target antigen (EGFR) on a target antigen-positive tumour cells, after which the ligand binds to and cross-links its cognate receptors on a neighbouring cell. This paracrine action also enables the elimination of target antigen-negative cells that possess functional TRAIL receptors. DISC, death-inducing signalling complex; EGFR, epidermal growth factor receptor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

Figure 5



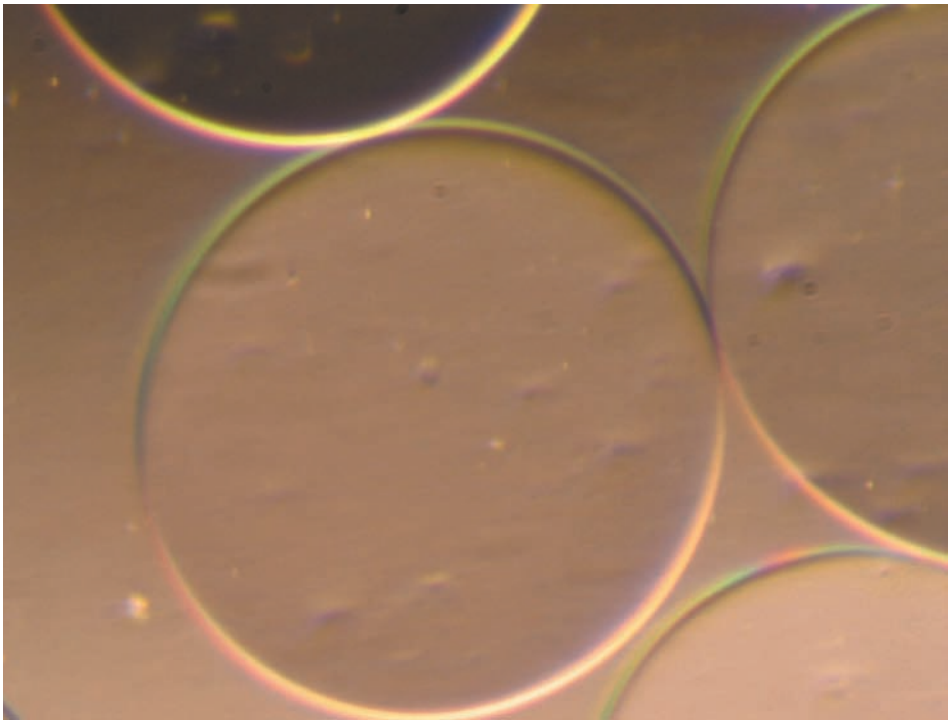
The anti-EGFR-TRAIL fusion protein has a twofold action on tumour cells; it induces the apoptosis signalling pathway and simultaneously inhibits the EGFR survival pathway. The fusion protein binds to the TRAIL receptor through its death-inducing domain (TRAIL) and at the same time binds to the EGFR through its targeting domain [scFv(EGFR)]. DISC, death-inducing signalling complex; EGFR, epidermal growth factor receptor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

Figure 2



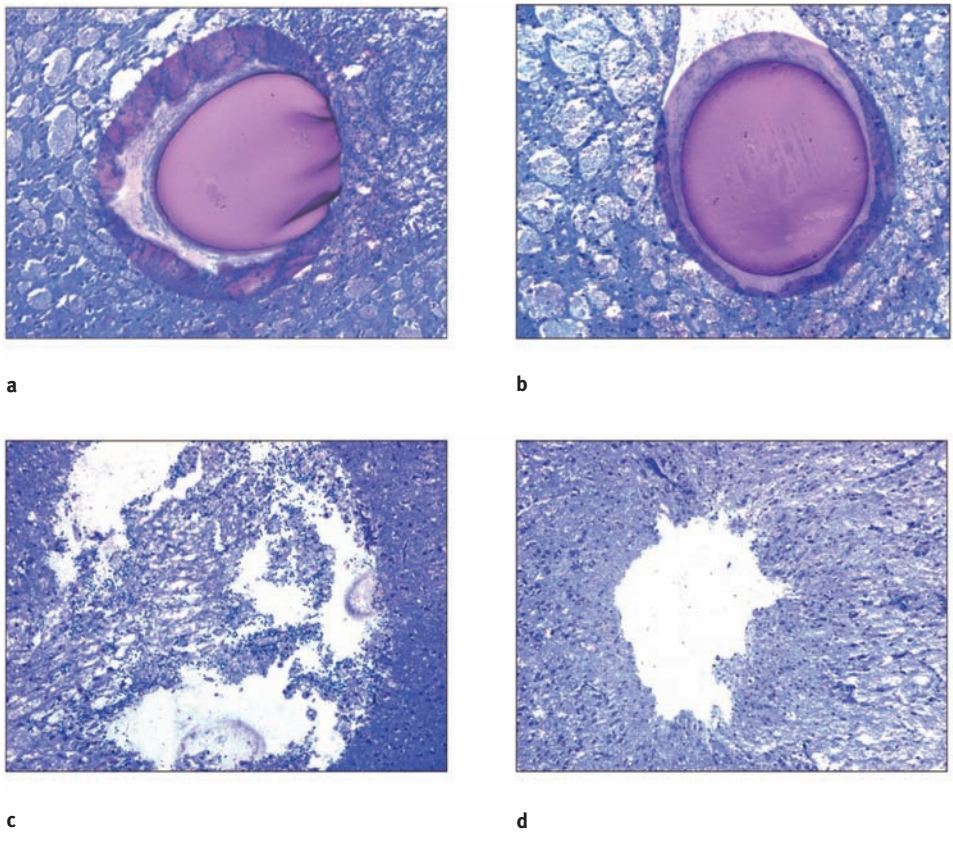
Immunohistochemical staining of primary glioblastoma tumor tissue for TRAIL (a), TRAIL-R1 (b) and TRAIL-R2 (c), within the intermediate zone is presented. Diffuse intracytoplasmic staining together with a ring of dark membranous staining (→) was found.

Figure 1



Empty alginate microcapsule (700 µm). Spherical and without imperfections.

Figure 2

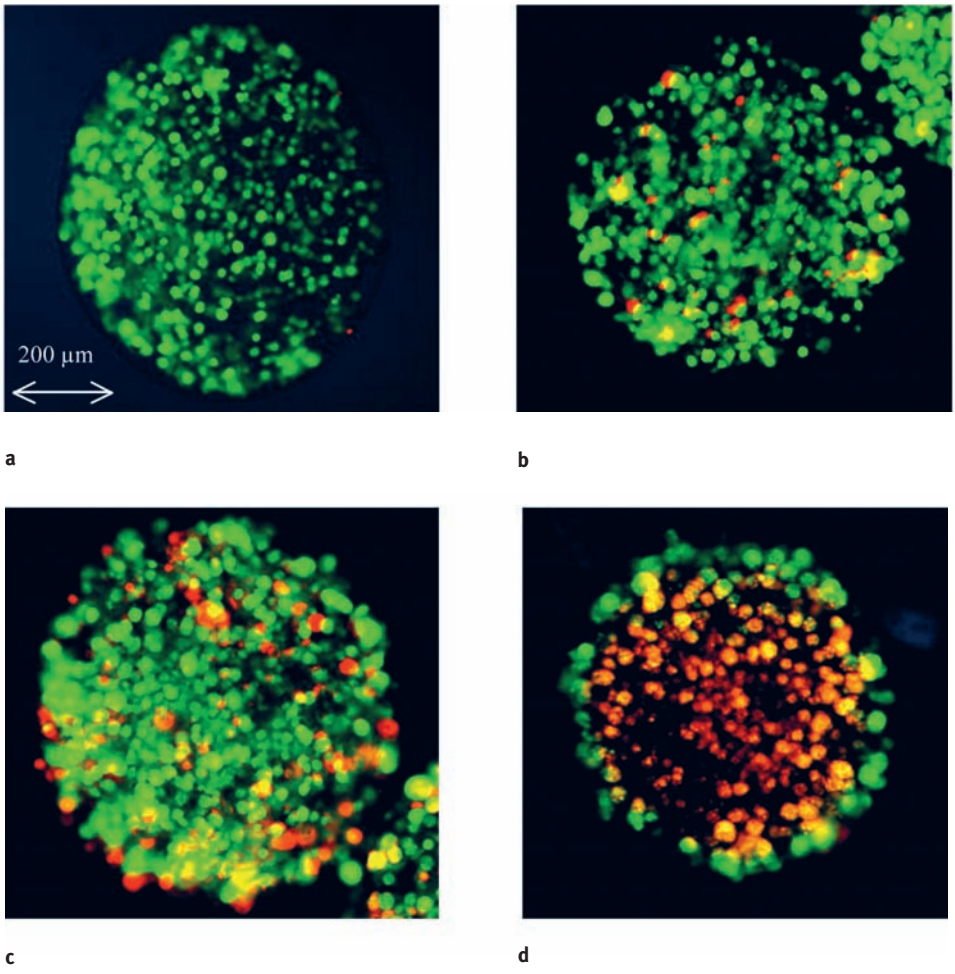


a **b**

c **d**

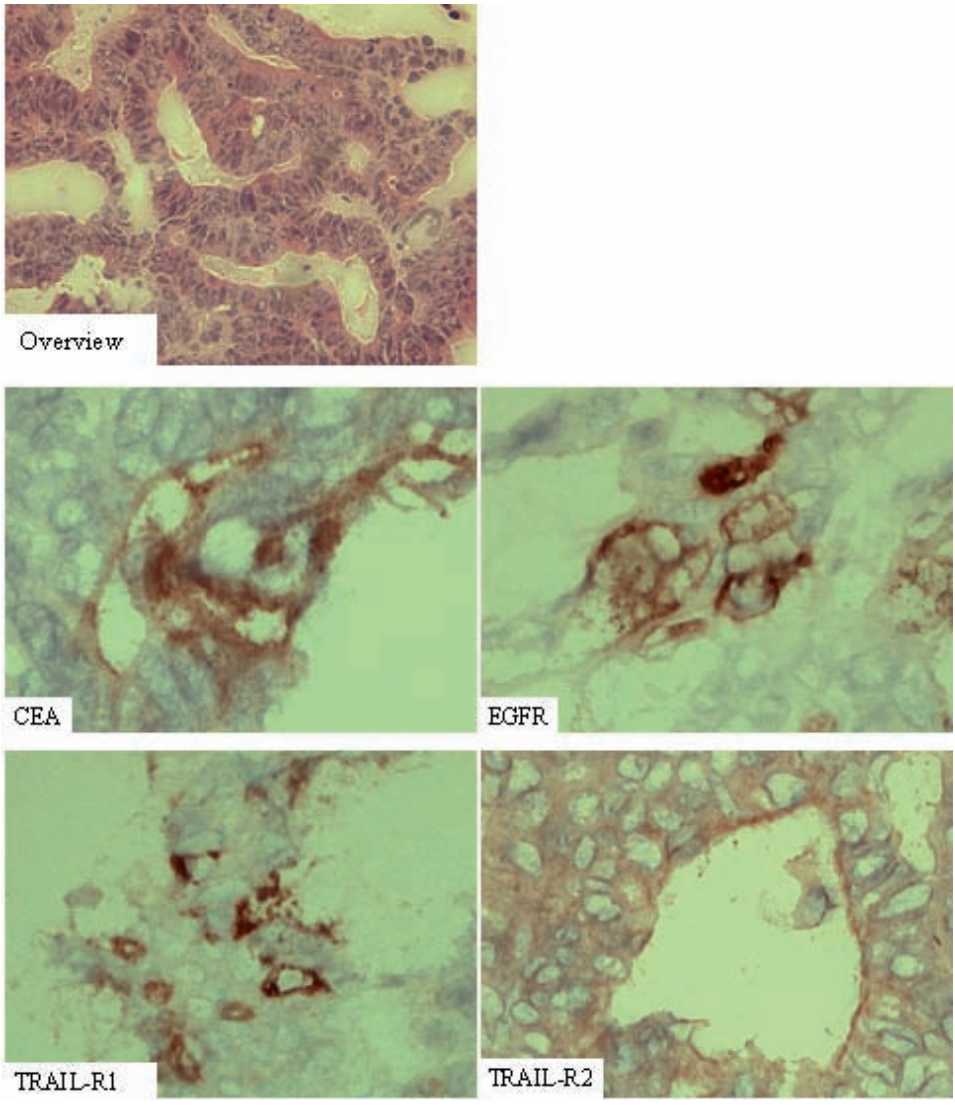
Mice brain sections stained with tonadine blue. Empty capsules (a) high guluronic, (b) intermediate guluronic (diameter capsule is 700µm) implanted in the cerebrum of a C75BL6 mouse.Sections were made 14 days after implantation. No sign of a foreign body reaction is visible in either high or intermediate-G capsules. (c) effect of a sham operation (sectioning and staining is performed 1 day post-implantation). (d) effect of residual trauma 14 days after the sham operation. The post sham cavity is reduced after 14 days. Immune cells are not detected on day 1 or day 14 after the sham operation.

Figure 3



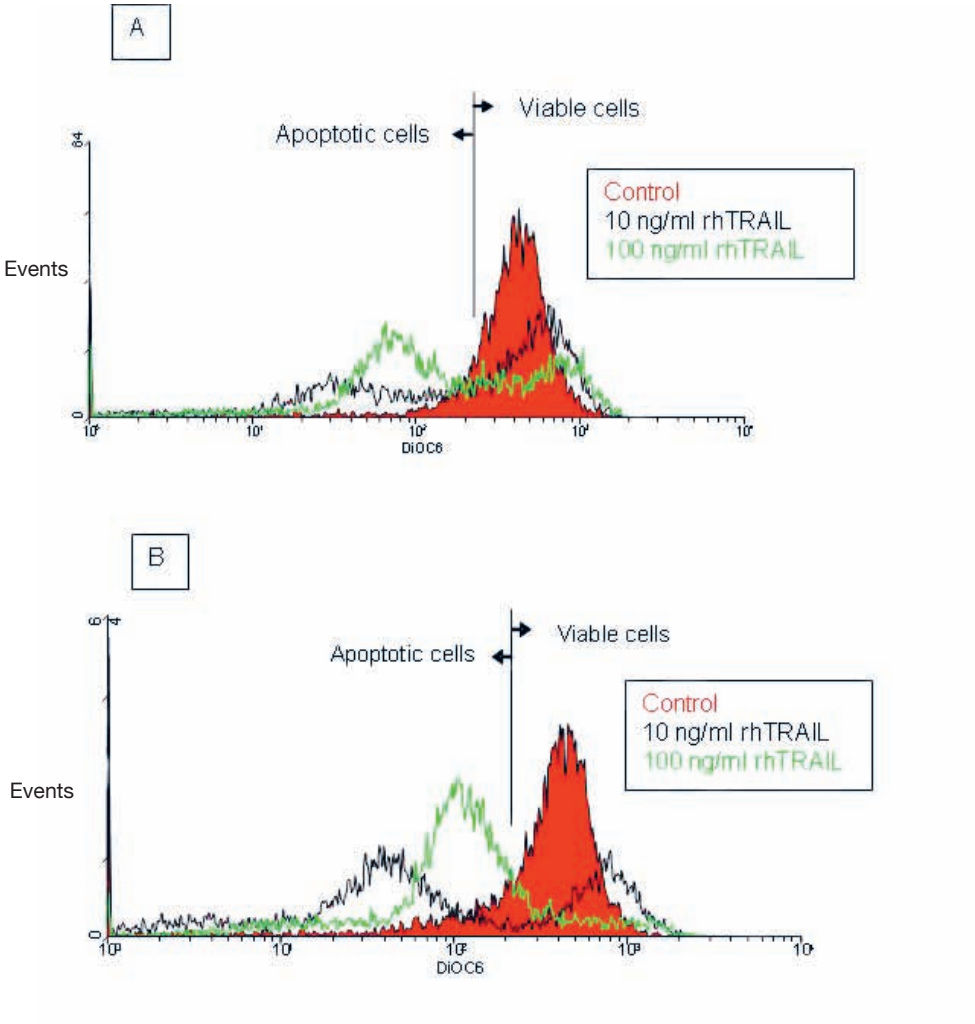
Fluorescent images of high guluronic acid alginate capsules (700 µm) with CHO-K1 (scFv425:sTRAIL) producer cells (2x107 cells/ml alginate). The capsules are stained with acridine orange (green) or propidium iodide (red). Viable cells emit green fluorescence, dead cells emit red fluorescence. (a) 1 day after encapsulation. (b) 3 days after encapsulation. (c) 16 days after encapsulation. There is an increase in cell death at day 16 in comparison with day 1 and 3. (d) 30 days after encapsulation. Cells in the center are dead/necrotic and living cells surround the central necrotic area.

Figure 6



Immunohistochemistry of an intracerebral SW948 xenograft. The SW948 xenograft overview (HE staining) shows the typical morphology of a colorectal adenocarcinoma. The xenograft expresses the colon embryonic antigen (CEA). EGFR, TRAIL-R1 and TRAIL-R2 expression is retained on the SW948 tumor 30 days after intracerebral inoculation.

Figure 2



Flow cytometric evaluation of DiOC6 staining, a measure for rapid apoptosis in non-irradiated (A) and irradiated A172 cells (B) either alone or after treatment with various concentrations of rhTRAIL. This figure is a representative of 2 independent experiments performed in triplicate.

Publication of this thesis was financially supported by: